OBSERVATIONS ON FOLIC ACID DEFICIENCY IN THE CHICK IN THE PRESENCE OF VITAMIN B_{12} [1]

C. A. NICHOL, L. S. DIETRICH, C. A. ELVEHJEM AND E. B. HART

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

SEVEN FIGURES

(Received for publication June 3, 1949)

Previous work has shown that chicks placed on a purified diet deficient in folic acid failed to grow normally and developed some degree of anemia. The administration of vitamin B_{12} or refined liver extract alone had no effect on hemoglobin regeneration (Nichol, Harper and Elvehjem, '49b) but did produce some growth response. When these chicks were kept on the folic acid deficient ration for several weeks following treatment with liver extract or vitamin B_{12}, they developed body tremors, extended, quivering wings and finally complete paralysis. None of these symptoms appeared in the control groups. Since the number of chicks used in the experiments

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by funds supplied by the Commercial Solvents Corporation, Terre Haute, Ind.; by Swift and Co., Chicago, Ill.; and by the Research Committtee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vitamin B_{12}, the vitamin B_{12} concentrate and crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid; to Abbott Laboratories, North Chicago, Ill., for haliver oil; to Wilson and Co., Inc., Chicago, Ill., for gelatin; to Allied Mills, Inc., Peoria, Ill., for soybean oil; and to E. I. du Pont de Nemours and Co., Inc., New Brunswick, N. J., for crystalline vitamin D.

[2] We are indebted to A. Sreenivasan for assistance in early preliminary work.

Copyright, 1949
The Wistar Institute of Anatomy and Biology.
All rights reserved.

on hemoglobin regeneration was limited, the growth of folic acid deficient chicks in the presence of vitamin B_{12} and the occurrence and cure of the observed symptoms were investigated further.

EXPERIMENTAL

Straight-run crossbred chicks (New Hampshire ♂♂ × Single Comb White Leghorn ♀♀), which were the progeny of hens fed diet B-1 [2] described previously (Robblee et al., '48), were used in all experiments. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied ad libitum. The chicks were wing-banded when one day old. Weights were recorded at weekly intervals.

The purified basal ration contained sucrose 61 gm, alcohol-extracted casein 18 gm, gelatin 10 gm, salts V 6 gm (Briggs et al., '43), soybean oil 5 gm, L-cystine 0.3 gm, thiamine hydrochloride 0.3 mg, riboflavin 0.6 mg, pyridoxine hydrochloride 0.4 mg, nicotinic acid 5.0 mg, calcium pantothenate 2.0 mg, biotin 0.02 mg, inositol 100 mg, choline chloride 150 mg, 2-methyl-1, 4-naphthoquinone 0.05 mg, α-tocopherol 0.3 mg, and fortified haliver oil (60,000 U.S.P units of vitamin A, 6,000 U.S.P. units of vitamin D_3 per gram) 40 mg.

Liver extract and vitamin B_{12} were compared with respect to their effect upon growth and the incidence of the symptoms (table 1). All chicks were fed the folic acid deficient basal ration for 19 days. Then one group received one U.S.P. unit of liver extract [3] by intramuscular injection on alternate days. Another group was fed a vitamin B_{12} concentrate mixed into the ration to provide approximately 3 μg of vitamin B_{12} per 100 gm of ration. A smaller group given no dietary supplement served as a negative control.

During a two-week period both liver extract and vitamin B_{12} stimulated the growth rate of the folic acid deficient chicks.

[2] Ground yellow corn 36 lb., ground wheat 25 lb., ground oats 10 lb., wheat bran 5 lb., wheat middlings 5 lb., alfalfa leaf meal 5 lb., fish meal 2 lb., soybean oil meal 2 lb., meat scraps 2 lb., bone meal 1 lb., iodized salt 0.5 lb., fish oil (85D-600A) 1 lb., $MnSO_4 \cdot H_2O$ 0.2 oz., riboflavin 100 mg.

[3] Lilly, Reticulogen, 20 U.S.P. units per milliliter.

Symptoms similar to those observed in previous experiments occurred in 54% of the chicks receiving liver extract and in 52% of those receiving vitamin B_{12}.

As the symptoms developed, the wings were first extended from the sides of the body and quivered persistently in the position shown in figure 1. A sudden rigid extension of the toes ocurred when the birds were picked up, and tremors of the whole body were noticed. The extension of the wings was periodic at first but became more pronounced and was accompanied by unsteadiness and difficulty in maintaining balance when walking (fig. 2). The birds consistently fell forward,

TABLE 1

The effect of liver extract and vitamin B_{12} concentrate on folic acid deficient chicks

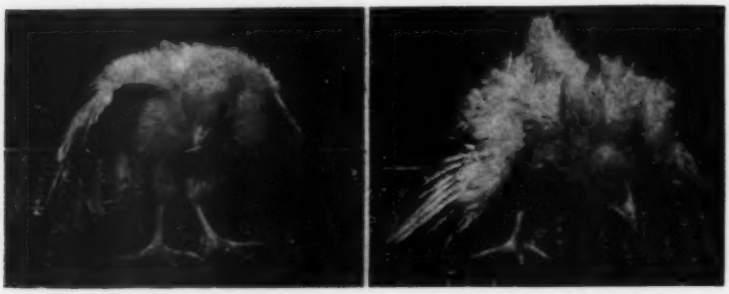
SUPPLEMENT	AVERAGE WEIGHT		NO. OF CHICKS	INCIDENCE OF SYMPTOMS
	19 days	33 days		
	gm	*gm*		%
None	70	79	14	0
Liver extract (0.5 U.S.P. unit per bird per day, injected)	71	115	83	54
Vitamin B_{12} concentrate, 0.7% (contributes 3 μg vitamin B_{12} per 100 gm of ration)	70	109	50	52

and in severe cases they would lie prostrate, apparently paralyzed. However, when the leg muscles were exercised and the bird was lifted to its feet partial recovery occurred. The chick could then stand and walk for some time, although the extended quivering wings persisted. The birds remained in a paralyzed condition for two to 4 days before dying.

The incidence of extended wings and paralysis was higher among the more rapidly growing birds within the groups which were supplemented with vitamin B_{12} alone. The chicks placed on test in order that the response of the symptoms to various treatments might be observed were generally 20 gm heavier than the average weight for the whole group.

The severity of the symptoms was graded on an arbitrary scale from zero (normal) to ++++ (prostrate), and on this basis appropriate groups of chicks were compared. The condition approximating grade +++ is shown in figure 2.

The response of the chicks to treatment with folic acid is shown in figure 3. Each curve represents the average grade of the number of chicks indicated inside the brackets. The control group showed a consistently severe condition; by the 13th



1 2

Fig. 1 (left) — Folic acid deficient chick receiving vitamin B_{12} (age — 5 weeks, weight 88 gm), showing the early periodic extension of the wings, which quiver when held in this position.

Fig. 2 (right) — Folic acid deficient chick receiving vitamin B_{12} (age — 5 weeks, weight 106 gm), showing the persistent extension of the wings and inability to maintain balance.

day, 4 of the 12 chicks in this group had died. All of the others survived the experimental period shown. Injection of 50 μg of folic acid caused complete recovery for 12 to 14 days, at which time the symptoms reappeared and progressed rapidly to a severe condition. Injection of 20 μg of folic acid caused marked improvement for 6 to 8 days. Fifty micrograms of folic acid administered orally were not as effective as 20 μg given by injection. All doses were administered only once, on the first day (indicated by zero) of the experimental period shown in figure 3.

Other substances tested which had no curative effect when administered orally included: *p*-aminobenzoic acid (25 mg/bird/day), ascorbic acid (50 mg/bird/day), thymine (2 mg/bird/day), adenine and guanine (2 mg of each/bird/day) and 60% methanol extract of fresh liver (0.3 ml = 3 gm fresh liver/bird/day). A concentrate of the *Leuconostoc citrovorum* factor [4] (Sauberlich and Baumann, '48), administered by injection, was also inactive (11,000 units/bird/day).

The growth-promoting activity of vitamin B_{12} was tested in the presence and absence of folic acid (table 2). Also the activity of a vitamin B_{12} concentrate mixed into the ration

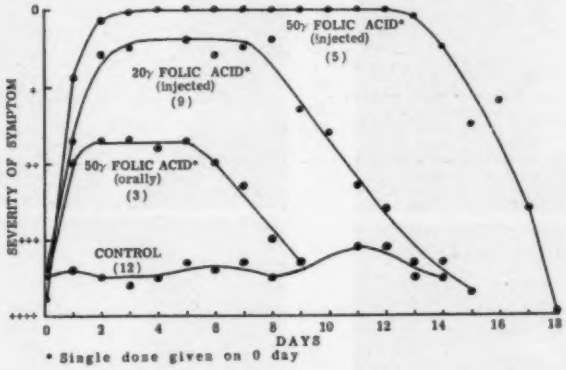


Fig. 3 Response of deficient chicks to treatment with folic acid.

was compared with that of pure vitamin B_{12} administered by injection. In the groups which were supplemented during the 4-week period, vitamin B_{12} caused a significant increase in the rate of growth when added to the folic acid deficient basal ration (groups 1 and 2) or when added to a ration containing 200 μg of folic acid per 100 gm (groups 3 and 4). Crystalline vitamin B_{12} (0.1 μg/bird/day injected intramuscularly during the third and 4th weeks) stimulated the rate of growth (group 6) to the same extent as the vitamin B_{12} concentrate which

[4] We are indebted to G. R. Drysdale and C. A. Baumann for a concentrate of the *Leuconostoc citrovorum* factor.

Fig. 4 No folic, no B_{12} (av. wt., 98 gm).

Fig. 5 No folic, plus B_{12} (av. wt., 166 gm).

Fig. 6 Plus folic, no B_{12} (av. wt., 286 gm).

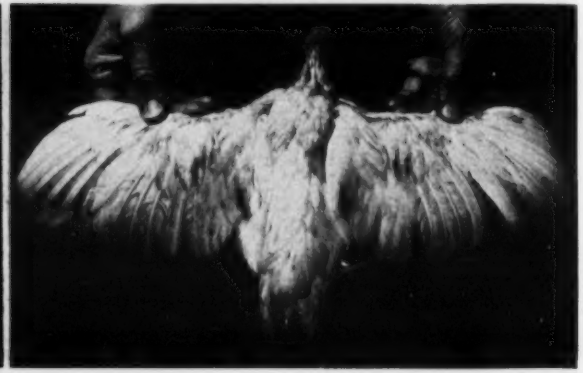


Fig. 7 Plus folic, plus B_{12} (av. wt., 340 gm).

Figs. 4–7 Typical feathering of chicks fed a purified ration with and without folic acid and vitamin B_{12} (age, 5 weeks).

was added to the folic acid deficient ration (group 5) during the same period.

The characteristic symptoms described above developed in all groups which received vitamin B_{12} without folic acid, whether as a concentrate mixed into the ration or as the crystalline material administered by injection. These symptoms were observed in 66% of the chicks in group 2, 52% in group 5 and 55% in group 6. None of these symptoms

TABLE 2

The effect of vitamin B_{12} when given with and without folic acid to chicks fed a purified ration

GROUP NO.	SUPPLEMENT	NO. OF CHICKS	Hb 4 WEEKS [1]	AV. WT. 4 WEEKS
			gm. %	gm.
1	None	15	7.26	75
2	B_{12} (concentrate, 0.7% ≡ 3 μg per 100 gm of ration)	17	6.74	128
3	Folic acid (200 μg per 100 gm of ration)	18	8.09	195
4	Folic acid (200 μg per 100 gm) + B_{12} (3 μg per 100 gm)	16	8.10	237
5	B_{12} (concentrate, 0.7% ≡ 3 μg per 100 gm of ration)[2]	71	6.64	94
6	B_{12} (crystalline, 0.1 μg per bird per day, injected)[2]	11	6.47	105

[1] Ten observations per group.
[2] Vitamin B_{12} given during the third and 4th weeks.

appeared in the negative control group (group 1) or in the groups which received folic acid (groups 3 and 4).

The effects of folic acid and of vitamin B_{12} upon feathering are compared in figures 4–7, which show the typical feathering of chicks in groups 1 to 4, table 2, at the age of 5 weeks. The retarded growth rate and poor feathering of the chicks receiving only the basal ration is characteristic of folic acid deficiency (Mills et al., '42). The addition to the folic acid deficiency ration of vitamin B_{12} alone increased

Fig. 6 Plus folic, no B_{12} (av. wt., 286 gm).
Fig. 7 Plus folic, plus B_{12} (av. wt., 340 gm).
Figs. 4–7 Typical feathering of chicks fed a purified ration with and without folic acid and vitamin B_{12} (age, 5 weeks).

the rate of growth but did not cause comparable improvement in feathering. When folic acid (200 μg/100 gm) was added to the basal ration, normal feathering was observed. Chicks which received both folic acid and vitamin B_{12} also showed normal feathering but grew more rapidly than the birds which were supplemented with folic acid alone.

DISCUSSION

Briggs et al. ('43) occasionally observed paralysis in chicks receiving the basal ration used in a study of vitamins B_{10} (for feathering) and B_{11} (for growth). They described the "trembling body and wings" and a temporary recovery from the paralyzed condition following exercise, which indicate the similarity to the symptoms described here. No effort was made at that time to secure chicks from a uniform source. The now-recognized variable growth response due to vitamin B_{12} stored by chicks from commercial sources was very likely a factor in the occurrence of the symptoms.

The early work on the separation of vitamins B_{10} and B_{11} was undoubtedly complicated because: (1) the occasional observation of the above symptoms indicated a lack of uniformity with respect to the vitamin B_{12} stored in the chicks; (2) varying amounts of vitamin B_{12} would occur in the fractions which were derived from solubilized liver; and (3) variation with respect to both growth and feathering would occur because of the marginal amounts of folic acid present in many of the fractions. The presence of two factors was clearly indicated by the data presented, but interpretation of the data was made difficult by inability completely to separate growth from feathering and by the different activities of the conjugated forms of folic acid in the microorganisms and in the chick.

Present knowledge of the activity of folic acid and vitamin B_{12} in the chick permits better interpretation of the earlier work on vitamins B_{10} and B_{11}. A purified ration supplemented with folic acid supports good growth and normal feathering. The addition of vitamin B_{12} and folic acid to the basal ration

also supports normal feathering, yet further increases the rate of growth. Chicks receiving the folic acid deficient basal ration showed the retarded growth and poor feathering earlier attributed to vitamin B_{10} deficiency. The addition of vitamin B_{12} to this basal ration increased the rate of growth but caused only slight improvement in feathering.

The occurrence of the extended wings and of paralysis was related to the growth rate induced by vitamin B_{12} in the folic acid deficient chicks. Richardson et al. ('45) have described a similar condition in turkey poults receiving a vitamin B_c (folic acid) deficient ration. The folic acid requirement of the more rapid-growing poult is reported to be at least twice that of the chick (Jukes et al., '47). The poults which showed spastic cervical paralysis and drooped, quivering wings had completely recovered one day following treatment with folic acid. Two observations of Richardson et al. ('45) bear important relationship to the work presented here: (1) more rapid development of cervical paralysis occurred when a liver extract containing only traces of folic acid was added to the cerelose-casein-gelatin basal ration; and (2) the symptoms developed several weeks sooner in poults hatched from hens without access to forage than in those from hens on range. The chicks used in the present studies were depleted by control of the hens' diet, and were the same as those which have shown consistent growth responses to treatment with liver extracts (Nichol et al., '49c) and crystalline vitamin B_{12} (Nichol, Dietrich, Cravens and Elvehjem, '49a).

In all experiments in which chicks receiving the folic acid deficient basal ration were supplied with vitamin B_{12} as the crystalline material, or with liver extract by injection or as a concentrate mixed into the ration, the characteristic symptoms described above developed in more than 50% of the birds. None of these symptoms appeared in the control groups. Treatment with adequate folic acid caused their rapid and complete disappearance.

The use of the crude charcoal concentrate of vitamin B_{12} left open the possibility of contamination with folic acid. In

the experiments here reported this vitamin B_{12} concentrate was compared with liver extract and crystalline vitamin B_{12}. The similar growth response and the consistent development of symptoms precludes explanation of the observed growth by the presence of folic acid.

The livers of severely depleted chicks were found to contain approximately one-half the concentration of folic acid that is present in the livers of normal birds. Any action of vitamin B_{12} resulting in more efficient utilization of folic acid within the tissues, or in stimulating the production of folic acid by intestinal microorganisms, should be reflected by changes in the growth rate and hemoglobin level. However, the increased growth was not accompanied by improvement in the anemia and poor feathering which are typical of folic acid deficiency in the chick. Also, folic acid caused disappearance of the symptoms characterized by the extended quivering wings before any change in hemoglobin level became apparent, and the high blood glucose observed in the chicks receiving the folic acid deficient basal ration was not lowered by the addition of vitamin B_{12} alone to the diet (Williams et al., '49). The mechanism by which vitamin B_{12} increases the growth rate may function independently of folic acid.

Although vitamin B_{12} has been shown to have animal protein factor activity (Ott et al., '48), other compounds possessing similar activity may exist. Smith ('48) has reported the purification of two pigments from liver which have antipernicious anemia activity. Several unidentified, microbiologically active components of liver extracts and fermentation products have been separated by means of paper chromatography (Cuthbertson and Smith, '49; Winsten and Eigen, '49). New and special conditions may be required to demonstrate the activity of these factors in the animal, but the possibility of their influence should not be overlooked.

SUMMARY

Folic acid deficiency in chicks fed a purified ration was accentuated by supplying vitamin B_{12} as the crystalline ma-

terial, by injection or as a concentrate added to the ration. Extended quivering wings, body tremors and complete paralysis developed in more than 50% of the chicks which received vitamin B_{12}. Treatment with folic acid caused complete disappearance of these symptoms within 24 hours. Injection of folic acid was more effective than oral administration. None of these symptoms developed in the control groups.

Vitamin B_{12} increased the rate of growth of the folic acid deficient chicks but did not cause marked improvement in feathering. The addition of vitamin B_{12} to a sucrose-casein purified ration containing 200 μg of folic acid per 100 gm caused a further significant increase in growth. The relation of folic acid and vitamin B_{12} to the previously described vitamins B_{10} and B_{11} is briefly discussed.

LITERATURE CITED

Briggs, G. M., Jr., T. D. Luckey, C. A. Elvehjem and E. B. Hart 1943 Studies on two chemically unidentified water-soluble vitamins necessary for the chick. J. Biol. Chem., *148:* 163.

Cuthbertson, W. F. J., and E. L. Smith 1949 Chromatography of the vitamin B_{12} group of factors. Biochem. J., Proc. Biochem. Soc., in press.

Jukes, T. H., E. L. R. Stokstad and M. Belt 1947 Deficiencies of certain vitamins as studied with turkey poults on a purified diet. J. Nutrition, *33:* 1.

Mills, R. C., G. M. Briggs, Jr., C. A. Elvehjem and E. B. Hart 1942 *Lactobacillus casei* factor in the nutrition of the chick. Proc. Soc. Exp. Biol. Med., *49:* 186.

Nichol, C. A., L. S. Dietrich, W. W. Cravens and C. A. Elvehjem 1949a Activity of vitamin B_{12} in the growth of chicks. Ibid., *70:* 40.

Nichol, C. A., A. E. Harper and C. A. Elvehjem 1949b Effect of folic acid, liver extract, and vitamin B_{12} on hemoglobin regeneration in chicks. Ibid., *71:* 34.

Nichol, C. A., A. R. Robblee, W. W. Cravens and C. A. Elvehjem 1949c The growth response of chicks to antipernicious anemia preparations. J. Biol. Chem., *177:* 631.

Ott, W. H., E. L. Rickes and T. R. Wood 1948 Activity of crystalline vitamin B_{12} for chick growth. Ibid., *174:* 1047.

Richardson, L. R., A. G. Hogan and H. L. Kempster 1945 The requirement of the turkey poult for vitamin B_{12}. J. Nutrition, *30:* 151.

Robblee, A. R., C. A. Nichol, W. W. Cravens, C. A. Elvehjem and J. G. Halpin 1948 The effect of hen diet and chick diet on the need of chicks for an unidentified growth factor. Poultry Sci., *27:* 442.

SAUBERLICH, H. E., AND C. A. BAUMANN 1948 A factor required for the growth of *Leuconostoc citrovorum*. J. Biol. Chem., *176:* 165.

SMITH, E. L. 1948 Purification of anti-anemia factors from liver. Nature, *161:* 638.

WILLIAMS, J. N., JR., C. A. NICHOL AND C. A. ELVEHJEM 1949 Relation of dietary folic acid and vitamin B_{12} to enzyme activity in the chick. J. Biol. Chem., *180:* 689.

WINSTEN, W. A., AND E. EIGEN 1949 Paper chromatography of vitamin B_{12} and related bacterial growth factors. Fed. Proc., *8:* 265.

THE PRODUCTION OF ANTIBODIES IN PROTEIN DEPLETED AND REPLETED RABBITS[1]

DOUGLAS G. GEMEROY AND ANNA H. KOFFLER
Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey

ONE FIGURE

(Received for publication March 10, 1949)

INTRODUCTION

The production of antibodies in the rabbit has been demonstrated over the years by various methods and with a wide variety of antigens. Little attention was paid to the diet in these studies, and the condition of the experimental animal was not considered in more than a general way. Of late years, however, our knowledge of nutrition has increased and its relation to immunity and resistance has been stressed (Cannon, '42). We have come to realize that the problem of antibody production is closely associated with the nutritional state of the animal.

Cannon, Chase and Wissler ('43) and Wissler ('47), using a low protein cookie plus carrots, have demonstrated that rabbits depleted in proteins on such a diet produced agglutinins of a much lower titer than rabbits on the usual commercial diet. These investigators studied the problem in depleted animals by means of agglutinin titers, which are end point determinations that test the relative concentration but not total antibodies of an antiserum.

For comparable quantitative studies, however, the full range of antigen antibody reactions must be considered (Boyden and

[1] These studies were supported by the Protein Metabolism Fund of the Bureau of Biological Research, Rutgers University, New Brunswick, N. J.

De Falco, '43; Boyden, Bolton and Gemeroy, '47), and unless the whole curve representing such reactions is taken into consideration, erroneous conclusions may be drawn with regard to the total antibodies produced. There is need to extend such studies to measure quantitatively the production of antibodies in rabbits at the end of an extended period of protein depletion, and again after repletion with a specific protein.

Since a simplified diet for the rabbit has not been formulated, it was necessary to try and develop a new diet that would maintain the animals through a lengthy depletion period as well as through the repletion period to follow.

METHODS AND MATERIALS

Since adult animals have been found to be better antibody producers than young ones (Freund, '30, '31) and are seemingly better able to withstand the rigors of long-term depletion, mature animals weighing around 3 kg and about three months old were used. These rabbits, of hybrid strains obtained from a local source, were conditioned for two weeks on a commercial diet prior to the start of the experiments.

Beef serum was used throughout the experiments as an antigen. The lyophilized beef serum [2] was dissolved in distilled water, Seitz-filtered, bottled under sterile conditions, labeled and stored in an icebox.

Many experiments at this laboratory [3] have shown that an intravenous injection of 1 ml of antigen given three to 4 weeks before the regular series is administered enhances the production of antibodies. Such a method we have called the presensitizing technique,[4] and the initial injection the presensitizing injection. The regular series consisted of 4 1-ml injections of the antigen, given subcutaneously every second day.

[2] Obtained through the courtesy of Sharp and Dohme, Inc., Research Laboratories, Glenolden, Pa.

[3] Unpublished data.

[4] Method suggested by Dr. R. J. DeFalco, Department of Zoology, Rutgers University, formerly with Sharp and Dohme, Inc., Glenolden, Pa.

One week after the last injection, blood was withdrawn from the ear artery in the required amounts. It was then stored in the icebox overnight, the sera being obtained by centrifugation next morning.

Tests for antibodies were carried out with these sera using the photronreflectometer, a photoelectric instrument which measures the turbidity developed in antigen antibody mixtures. In this method the antibody is kept constant and the antigen is diluted. The amount of precipitate formed is correlated with the readings on the galvanometer scale; thus the greater the antibody content of a serum the greater the precipitate and the higher the galvanometric reading (Libby, '38; Boyden and DeFalco, '43). Curves relating the antigen dilution with the galvanometric reading were plotted, the curve areas being a measure of the total turbidity of such reactions.

Total serum protein and non-protein nitrogen, hemoglobin and hematocrit determinations were carried out every three weeks throughout the experiment.

Preliminary experiments

In their search for an adequate depletion diet for rabbits Cannon and his co-workers ('43) developed a low protein cookie, to be supplemented by carrots. One hundred grams of cookies were given to adult animals every other day, supplemented by 100 gm of carrots on the alternate day. Although the rabbits usually consumed their carrots, only about 60% of the cookies were eaten (Wissler, '47).

In our preliminary experiments, 100 gm of carrots were given daily and 100 gm of a modified Cannon cookie every other day to deplete our animals. This modified cookie consisted of the following ingredients: dextrose and dextrin, each 18.5%; cornstarch 40%; commercial hydrogenated cottonseed oil,[5] 3.5%; cellu flour 15%; Wesson salts ('32) 4.5%. One hundred grams of these cookies contained 0.054 gm of nitrogen.

[5] Crisco.

On this cookie-plus-carrot diet the rabbits lost weight rapidly. With the continuation of the diet the animals became extremely weak and death resulted in several cases. When depletion studies are carried out, it is important to distinguish between a diet with a single factor lacking and one where multiple deficiencies exist. Thus, in the series discussed above, the diet was deficient in both proteins and vitamins. To eliminate a possible vitamin deficiency, the following vitamins were added to every 100 gm of the diet: thiamine 400–500 μg; pyridoxine HCl 500–600 μg; calcium-*d*-pantothenate 4 mg; niacin 10–12 mg; *l*-inositol 36 mg; choline 100 mg; riboflavin 600–700 μg; Navitol[6] 1,665 I.U. vitamin A, 333 I.U. vitamin D. Since many of the vitamin requirements of the rabbit have not yet been studied adequately, the amounts used were based on the known vitamin requirements of the dog, rat and guinea pig, modified according to what was thought might best meet the vitamin requirements of the rabbit.

The feeding procedures were changed slightly, and instead of every second day, 100 gm of carrots were fed daily. With these changes, 4 animals were carried successfully through a three-months' depletion period. Using the cookie type of diet and substituting 15% casein for an equivalent amount of carbohydrates, we were, however, unable to replete them with equal success. Therefore a search was made for an uncooked diet palatable to rabbits and on which they could be depleted and repleted successfully. The aim was to develop a completely purified diet, but this was not achieved since the carrot supplement still had to be used. Without the carrots even this diet was not acceptable to most of the experimental animals.[7]

To maintain the animals in a good state of health upon depletion and repletion, a new diet was introduced, patterned

[6] Squibb.

[7] However, in experiments conducted since, 4 rabbits were maintained over a 6-week period on the 24% casein diet to which 3% alfalfa meal was added, with no supplement of carrots.

after the one developed by Allison and Anderson ('45) in their depletion studies of the dog. After many trials with varying amounts of agar and cellu flour, the diet described in table 1 was devised; it was readily consumed by the rabbits. They ate on the average 75 gm of this diet per rabbit per day during the depletion period, along with an average of 135 gm of carrots. This diet was also fortified by the vitamins listed above. Water was given ad libitum.

Preparation of the diet

The diet is prepared as follows: the agar is dissolved in boiling water and commercial hydrogenated cottonseed oil [8] is melted into the solution. The dry ingredients are weighed and thoroughly mixed in a separate pan and added to the agar-cottonseed oil mixture after it has cooled down to about 50°C. This facilitates mixing and the production of an even-textured diet.

The mixing is carried out with an electric stirrer at high speed until a homogeneous mixture is obtained. When cooled down sufficiently, and before solidifying, the water-soluble vitamins are added.[9] The resulting substance is then poured into a pan and stored in the icebox. A solid diet results. Small pieces in the amounts required for feeding purposes can be readily cut. Navitol, one drop per 100 gm of diet, is added to each ration at the time of feeding

Caloric intake during protein depletion

Careful records were kept of the daily amounts of food consumed by the rabbits. The caloric value of the low protein diet is given in table 1; the caloric value of 100 gm of carrots was estimated at 40 Cal.

The average caloric intake during the depletion period, as calculated per kilogram of body weight per day, is presented

[8] See footnote 5, page 301.

[9] In our previous diets the water-soluble vitamins were added, with the Navitol, on top of the ration. By adding these vitamins directly to the diet during its preparation, a better distribution is assured.

in table 2. This table also shows the weight losses suffered during this period, the average length of which was 98 days. During this time the rabbits consumed, on the average, 75 gm of diet and 135 gm of carrots per animal per day.

TABLE 1

Low protein diet[1]

INGREDIENTS	DIET COMPOSITION	
	gm %	*gm*
Dextrose	19.50	390
Dextrin	19.50	390
Cornstarch	42.16	843
Crisco	3.69	74
Cellu flour	7.38	148
Wesson salts	4.61	92
Agar	3.16	63
Total of dry ingredients	100.00	2,000
Water-plus-vitamin solution		2,800
Total		4,800

[1] One hundred grams of this diet contained 70 Cal. and 0.054 gm of nitrogen by micro Kjeldahl analysis.

TABLE 2

Average caloric intake per kilogram body weight and weights during the depletion and repletion periods

RABBIT NO.	DEPLETION			REPLETION	
	Ave. Cal. intake per kg body weight	Weight		Weight	Ave. Cal. intake per kg body weight
		Start	End	End	
		gm	*gm*	*gm*	
41	68.1	3,540	3,050	3,400	75.2
42	44.7	3,325	2,300	2,700	82.3
44	60.0	3,410	2,260	2,870	82.7
45	60.0	3,310	2,550	2,960	84.7
47	62.5	3,140	2,470	2,910	83.3
60	53.0	3,710	3,340	3,280	79.1
61	68.2	3,210	2,210	2,140	78.4
63	66.5	3,090	2,630	2,960	86.8
65	70.9	3,540	2,630	2,910	93.4
	Ave. 61.5				Ave. 82.9

Repletion diet and caloric intake during repletion

Once this type of purified diet with an agar base was developed, it was easy to convert it into a repletion diet simply by substituting 24% casein for an equivalent amount of carbohydrates. The composition and caloric value of the protein repletion diet are given in table 3. This diet was enriched by vitamins in the same way as the depletion diet, and was also supplemented by carrots. The animals ate it readily, and in

TABLE 3

High protein diet [1]

INGREDIENTS	DIET COMPOSITION	
	gm %	*gm*
Dextrose	13.61	272
Dextrin	13.61	272
Cornstarch	30.21	604
Crisco	3.53	71
Casein [2]	25.15	503
Cellu flour	6.82	136
Wesson salts	4.13	83
Agar	2.94	59
Total of dry ingredients	100.00	2,000
Water-plus-vitamin solution		2,800
Total		4,800

[1] The caloric value of this diet was 70 Cal. per 100 gm.

[2] Borden's vitamin-free Labco casein was used throughout the experiment. Average protein content: 82%.

experiments since conducted at our laboratories we have had little difficulty in feeding this 24% casein diet to rabbits even after they have been for many months on a commercial stock diet.

The average length of the repletion period was 37 days. During this time the rabbits consumed, on the average, 150 gm of diet and 100 gm of carrots per rabbit per day. The average caloric intake per kilogram of body weight per day was 82.9 Cal., as compared with 61.5 Cal. during the depletion period (table 4).

Three of the rabbits were kept for an additional period of 47 days on the 24% casein diet plus carrots. Their food and caloric intake ran close to the data reported above. During this additional 47-day period the animals consumed, on the

TABLE 4

The effects of diets on antibody response curve areas

RABBIT NO.	AT THE END OF DEPLETION	AT THE END OF REPLETION
	Animals on depletion and repletion	
	GROUP I	
41	1,099.5	2,363.0
42	114.5	726.5
44	87.0	360.5
45	131.5	466.0
47	498.0	1,859.0
	GROUP II	
60	389.5	1,464.5
61	186.0	872.0
63	891.0	2,530.0
65	824.5	2,398.5
	Ave. 469.1	Ave. 1,448.9
	Increase due to high protein diet	
	Animals on commercial diet	
70	296.5	276.0
71	1,286.5	1,890.0
72	184.5	308.0
73	458.5	649.0
74	244.0	84.5
	Ave. 494.0	Ave. 641.5
	Increase due to multiple injection series	30.0%

average, 150 gm of diet and 115 gm of carrots per rabbit per day and the average caloric intake was 89.4 Cal. The animals gained weight steadily during this period and seemed to be in a good nutritional state when the experiment was discontinued.

These data seem to point to the 70 to 80 Cal. requirement of the rabbit per kilogram of body weight per day, which is in close agreement with the requirements already established for the dog and the rat.

RESULTS

Effects of protein depletion and repletion upon serum protein and hematological values

These values were measured every three weeks during the experimental period. As a general rule the concentration of serum proteins decreased as depletion progressed, though this was not as marked in some animals as in others. Hematocrit and hemoglobin values, however, had fallen to a greater degree in all the animals by the end of the depletion period. After 5 weeks' repletion the serum protein levels were higher than at the start of the experiment; hematocrit values were also higher in most cases, but hemoglobin values were still below those orginally recorded in all but two cases. A 24% casein diet does not seem to be adequate for hemoglobin regeneration in rabbits over such a short repletion period.

Antibody response in depleted and repleted rabbits

Figure 1 and table 4 show the effects of protein depletion and repletion on antibody production when beef serum was used as an antigen. One surprising result was observed with the animals depleted in proteins for a period of 98 days; even after this long depletion period some of the rabbits still produced very good antibodies (fig. 1-A). In fact, the curve areas representing the turbidities of the antigen antibody reactions are greater in some cases than those for the animals fed the usual commercial diet. When the depleted animals were repleted with the 24% casein diet plus carrots for a period of 5 weeks, the antibody response markedly increased (fig. 1-B). The differences in the curve areas obtained under similar experimental conditions show the wide variation of individual response that may be found in rabbits.

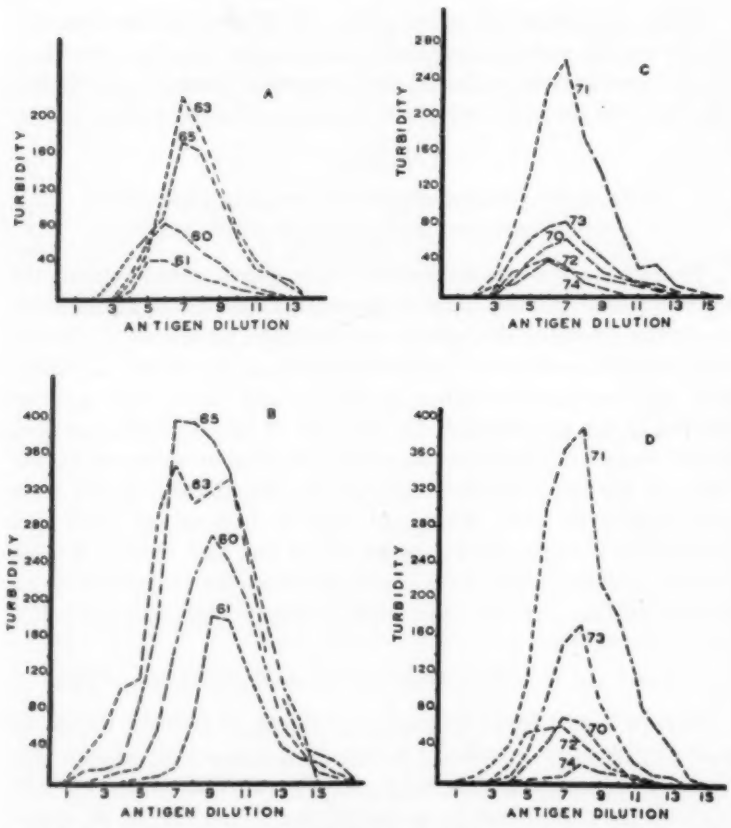


Fig. 1-A Antigen-antibody precipitation curves obtained with 4 protein depleted rabbits with vitamin supplements added to the diet (group II).

Fig. 1-B Antigen-antibody precipitation curves obtained with 4 rabbits repleted on a 24% casein diet for 37 days after a 97-day period of protein depletion (group II).

Fig. 1-C Antigen-antibody precipitation curves obtained with 5 rabbits fed a commercial diet, after a single injection series with beef serum.

Fig. 1-D Antigen-antibody precipitation curves obtained with 5 rabbits fed a commercial diet following a second series of injections with beef serum.

Note: Antigen dilution values represent the amount of protein in the antigen. The original dilution, marked "1" on the dilution scale in all figures, is one part of protein to 62.5 parts of saline. Turbidity is a measure of the antigen antibody reactions in terms of the galvanometric scale of the instrument used. The numbers on the curves identify individual rabbits used in the experiments.

It is generally agreed that a double injection series produces more antibodies than a single series; athough this is not always the case, as is shown with respect to two of the rabbits fed a commercial diet. To see to what extent a double series of injections was responsible for the increase in antibodies, 5 rabbits fed a commercial diet were injected in the same manner and over the same period of time as the experimental animals. The increase due to the multiple injections series was 30.0% (fig. 1-C, 1-D), whereas the increase due to the high protein diet was 209.0% (table 4). Thus the diet was primarily responsible for the enhanced antibody production.

As was previously stated, all diet factors must be taken into consideration in depletion and repletion studies where antibody production is being measured. In our preliminary experiments the diet was deficient in vitamins as well as in proteins, and with the group of rabbits fed this diet our poorest antibody response was obtained. With the animals in groups I and II (table 4) whose diet was fortified with what might be considered the essential vitamins, the antibody response was much greater, indicating the important role of vitamins in antibody production. Stoerk and Eisen ('46) and Axelrod et al. ('47) have shown that a deficiency of pyridoxine and other vitamins in the diet markedly decreases the amount of circulating antibodies.

The value for antibody production of an adequate protein intake, which is here demonstrated, has been stressed before (Cannon et al., '43; Clausen, '34). It would seem, however, that if all other dietary constituents except protein are adequate, the rabbit is still able to produce good antibodies, as is shown in figure 1-A.

These results seem to indicate that a high-protein diet fortified with other essential constituents may be of great practical importance in the production of hyperimmune sera. Further studies on the relation of diet to antibody production are under way.

SUMMARY

1. Antibody response in protein depleted and repleted rabbits to beef serum as antigen was quantitatively studied by means of the precipitin reaction.

2. Turbidity measurements of the precipitins formed over the whole reaction ranged were carried out on the Libby photronreflectometer.

3. The injection technique used for antibody production consisted of a presensitizing dosage of 1 ml of antigen given intravenously and followed by a regular series of 4 subcutaneous injections of the same amount of antigen on alternate days, three to 4 weeks later.

4. A new type of semi-purified diet in an agar base was developed that would carry rabbits through the depletion and repletion periods. This diet was supplemented by carrots throughout the experiments.

5. The average caloric intake of the rabbit was determined during the course of the experiments.

6. Antibody response is higher in repleted than in depleted animals. It is only slightly higher in rabbits fed a commercial diet than in the protein depleted animals.

7. A high protein diet supplemented by other essential dietary factors is valuable for the production of hyperimmune sera in rabbits.

LITERATURE CITED

ALLISON, J. B., AND J. A. ANDERSON 1945 The relation between absorbed nitrogen, nitrogen balance and biological value of proteins in adult dogs. J. Nutrition, *29:* 413.

AXELROD, A. E., B. B. CARTER, R. H. MCCOY AND R. GEISINGER 1947 Circulating antibodies in vitamin deficiency states. I. Pyridoxine, riboflavin and pantothenic acid deficiencies. Proc. Soc. Exp. Biol. Med., *66:* 137.

BOYDEN, A., E. B. BOLTON AND D. GEMEROY 1947 Precipitin testing, with special reference to the photoelectric measurment of turbidity. J. Immun., *57:* 211.

BOYDEN, A., AND R. DEFALCO 1943 Report on the use of the photronreflectometer in serological comparisons. Physiol. Zool., *16:* 229.

CANNON, P. R. 1942 Antibodies and the protein-reserves. J. Immun., *44:* 107.

CANNON, P. R., W. E. CHASE AND R. W. WISSLER 1943 The relationship of the protein-reserves to antibody production. I. The effects of a low-protein diet and of plasmapheresis upon the formation of agglutinins. Ibid., *47:* 133.

CLAUSEN, S. W. 1934 The influence of nutrition upon resistance to infection. Physiol. Rev., *14:* 309.

FREUND, J. 1930 The influence of age upon antibody production. J. Immun., *18:* 315.

———— 1931 Reaction of young and adult rabbits to pneumococci injected into the skin. J. Exp. Med., *54:* 171.

LIBBY, R. L. 1938 The photronreflectometer — an instrument for the measurement of turbid systems. J. Immun., *34:* 71.

STOERK, H. C., AND H. N. EISEN 1946 Suppression of circulating antibodies in pyridoxine deficiency. Proc. Soc. Exp. Biol. Med., *62:* 88.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, *75:* 339–340.

WISSLER, R. W. 1947 The effects of protein depletion and subsequent immunization upon the response of animals to pneumococcal infection. I. Experiments with rabbits. J. Inf. Dis., *80:* 250.

EROSION OF MOLAR TEETH BY ACID BEVERAGES [1,2]

C. M. McCAY AND LOIS WILL
Animal Nutrition Laboratory, Cornell University, Ithaca, New York

(Received for publication June 24, 1949)

The erosion of the teeth by acids contained in such foodstuffs as lemon juice has long been recognized (Pickerill, '12). McClelland found he could decalcify teeth if they were left in a solution at a pH of 4.5 for 12 hours (McClelland, '26). In regard to more acid media he stated, "Certainly the presence of a reaction of 3.5 and below, even if existing for only a few minutes, is a potential source of damage to the teeth."

The great increase in the consumption of cola beverages having a pH of 2.6 during the past 30 years has renewed interest in the effect of acids upon teeth. McClure ('43) in the case of rats and Stafne and Lovestedt in the case of man ('47) have called attention to the erosion of both the enamel and dentine by such acid beverages.

Wynn and Haldi ('48) have produced erosion of the enamel of the teeth of rats fed fruit juices that had only about one-tenth the hydrogen ion concentration of cola beverages. Their animals were fed the juices for long periods of 100 to 200 days. If the amounts they fed were related to the body weight of a man, their studies were equivalent to having a man ingest one-half to one ton of fruit juice during a period of 200 days.

[1] These studies were made under a grant from the Office of Naval Research, Contract N6ori–91, Task Order 6 (NR 182–077). Special assistance was given by Comdr. C. A. Schlack (D.C.) and his staff at N.M.R.I.

[2] Substantial technical assistance was rendered by Mrs. Audrey M. Ackerman and Janet S. Ricker.

In an earlier report (Gortner, McCay, Restarski and Schlack, '46) the effect of oxalate in preventing the acid erosion of teeth was described. Many other factors, such as the buffering capacity of the saliva, may serve to help prevent this erosion. Smith ('46) has found that even emotional conditions may play a part in the control of acid erosion by influencing the volume and composition of saliva.

The present study covers a series of experiments on acid erosion and its prevention. Rats were used, and the same techniques described earlier (Gortner et al., '46) of autoclaving and scoring teeth (Restarski, Gortner and McCay, '45), were employed.[3] A question has been raised concerning the suitability of the rat for the study of tooth erosion, since this animal may drink in a manner different from that of man. However, some earlier studies were made with monkeys and dogs (Bieri, McCay, Restarski and Gortner, '46) and erosion was similar in these species to that found in the rat. Monkeys are known to drink like man and have been taught to drink from a cup.

RESULTS AND DISCUSSION

Minimum detectable erosion

The degree of acid erosion is the result of several factors; namely, the hydrogen ion concentration of the solution, the titratable acidity, the buffer action within the mouth, and the type of anion.

Since the national consumption of soft drinks is now claimed to have a retail value in excess of $700,000,000, a series of such drinks were analyzed at the Naval Medical Research Institute, but the analyses were not published. The cola beverages were found to be among the most acid and to contain 10% surcrose and 0.055% phosphoric acid with a pH of 2.6. In our earlier comparative tests it was found that the degree of erosion of the molars was identical in experimental

[3] The stock diet used in all these studies was the open formula dog feed made by the Grange League Federation, Canandaigua, New York.

animals given cola beverages from dispensing machines and in those given a solution of phosphoric acid (0.055%) and sucrose (10%) made with distilled water. In order to determine the minimum amount of this acid solution that would erode the teeth of rats, varying amounts were fed and the animals were killed at intervals starting after the first day. Control rats were given distilled water instead of the acid beverage.

Detectable erosion was produced when a rat had consumed 10 ml of the acid during 24 hours, or 5 ml daily for two days. A few organic acids in a 10% sucrose solution were similarly tested. Erosion could be detected after three daily feedings of 3 ml of 0.23% tartaric acid. Similarly, the effects of a single daily ingestion of two, three or 5 ml of 0.58% citric acid could be observed. Rats often refused to drink 4% solutions of acetic acid, even if the solution contained 20% sucrose. In a few cases, however, erosion was found after the ingestion of 35 ml of such solutions.

Brown deposits on both sides of all molars were observed in 5 rats of the 17 that were used in the acetic acid studies. These brown deposits have been observed in the course of various experiments. It is believed that they are due to something in the mineral mixtures fed the animals, but their exact cause is still unknown.

The effects of long-continued ingestion of acid beverages

In order to compare the effects of long-continued ingestion of phosphoric acid, in the concentration used in cola beverages, with those of natural juices, 4 groups of rats were selected at an age of 50 to 60 days. Each rat was fed a daily allowance of 10 ml of the substance being tested. One group of rats received orange juice, one tomato juice, one phosphoric acid-sucrose in the concentrations used in cola beverages, and one distilled water. Rats were killed at the end of 6 weeks, 4 months and 6 months.

At the end of 6 weeks no erosion could be detected in the teeth of the animals given distilled water or tomato juice. Some erosion was found in the rats fed orange juice, and the most extensive erosion was found in those fed phosphoric acid. At the end of 4 months the erosion was more marked in those given orange juice or phosphoric acid, with some etching of the molars in those fed tomato juice. After 6 months the erosion produced by the phosphoric acid solution was so severe that it could not be graded in the usual manner. The tops of the molars were only slightly above the gum line.

In all cases erosion by tomato juice was the least, with phosphoric acid exerting the greatest effect upon the teeth.

Rate of erosion of teeth in phosphoric acid solutions

In order to have an approximate value for the rate of erosion of human teeth in phosphoric acid — sugar solutions similar in composition to cola beverages, two series of trials were made by immersing extracted human teeth in (a) solutions containing 0.055% phosphoric acid and 10% sucrose, and (b) a cola beverage purchased from a dispensing machine in the local market. The roots of the teeth were first coated up to the gum line with paraffin.

The teeth were then placed in flasks containing 50 ml of either phosphoric acid solution or cola beverage. The solutions were analyzed for calcium after definite time intervals. The flasks were not shaken.

The data are summarized in table 1. Although they might seem to indicate a greater rate of erosion in the cola beverage, this is probably not significant due to variability among extracted teeth and the difficulty of selecting matched specimens.

The buffer capacity of the mouth

Numerous studies, such as those of Pickerill ('12), have been made of the titratable acidity of saliva after stimulation by various foods. Also, in recent years many reports have been made of the pH of saliva under various conditions.

Little attention, however, has been paid to the hydrogen ion concentration of the mouth after stimulation by acid beverages. Since two important factors in the erosion of teeth are pH and titratable acidity, it seemed worthwhile to measure the buffer action of the mouth.

A simple test was devised for this purpose. A solution similar to cola beverages in its content of acid and sugar was prepared with 10% sucrose and 0.055% H_3PO_4. Varying amounts of this solution were tested and 10 ml were found the most satisfactory to hold and agitate in the mouth for a half

TABLE 1

Rate of solution of calcium from human teeth immersed in phosphoric acid or cola beverage

TIME IN SOLUTION	MEAN WT. TOOTH		DISSOLVED	
	0.055% H_3PO_4	Cola	0.055% H_3PO_4	Cola
hours	*gm*	*gm*	*mg Ca/gm tooth*	
3	1.20	1.35	1.3	1.4
6	1.47	1.24	1.9	2.5
9	1.01	1.23	2.7	4.4
12	0.88	0.98	6.0	4.2
24	1.34	0.68	3.9	6.3
72	1.18	0.60	5.3	11.7
120	1.42	1.42	5.5	5.6
188	1.57	0.91	6.0	9.0
336	1.55	0.67	6.7	14.6

minute. After this mixture of solution and saliva was returned to a beaker, the pH was determined by using the glass electrode and Bogen's Universal Indicator. Both methods proved satisfactory.

This test has been made on several hundred individuals and repeated frequently, under varying conditions, on a selected group. The range of pH values found varies from 2.8 to 5.6. Among a group of 32 young naval officers, composed half of women and half of men, the mean value for the men was 4.2 and for the women 3.7. Among a group of 20 graduate students composed of 8 women and 12 men the mean values

were 3.8 for the women and 4.4 for the men. Many tests have indicated this modest difference between the sexes. It is not related to height or body weight.

This buffer power of the mouth in the presence of acid beverages remains quite constant throughout the day. The results of studies made in relation to time of eating are summarized in table 2.

Teeth are usually considered subject to erosion if the pH of the mouth is lower than 3.5. Since the mouth in some cases cannot buffer an acid beverage to this extent even after a half minute and with a modest amount of acid, it would seem possible that some individuals have conditions in their mouths which would lead readily to acid erosion.

Our laboratory has made no study of the teeth of heavy consumers of acid beverages. However, in the course of testing the buffering capacity of the mouth it has proved very difficult to continue studies upon the same individuals if testing is on consecutive days. After a few days the subjects develop such sensitive teeth that they have difficulty in chewing food, and hence dislike to continue as members of the group being studied.

The oxalate film

Since Restarski (Gortner et al., '46) first observed an oxalate film on the teeth of rats whose diet contained small amounts of sodium oxalate, many questions have been raised concerning the formation and permanency of this coating. Seven experiments were run, employing 204 young rats, to determine the effect of feeding various levels of sodium oxalate, mixed with the diet or dissolved in an acid beverage.

When rats were fed a finely ground meal containing 0.2 or 0.3% sodium oxalate as a fine powder for 14 days and then allowed to drink 20 ml of cola beverage, there was partial protection inasmuch as islands of enamel remained without the usual etching down to the gum line. However, erosion was marked around these coated areas where the deposit had formed.

In order to study the deposition of the film, a meal diet was mixed to contain 10 mg of sodium oxalate per 10 gm of dry feed. This allowance was fed daily to each rat in a group for a period of 13 days. Individuals were killed starting after the first day, which allowed the ingestion of at least 10 mg of sodium oxalate.

In a few cases thin patches were observed after this first day. After two days and the ingestion of 20 mg of oxalate,

TABLE 2

The buffer capacity of the mouth before and after eating (pH after holding acid beverage in mouth for 30 sec.)

SUBJECT	P	M	D	G	L	M	S
				Women graduate students			
1/2 hr. before breakfast	3.9	2.9	3.0	4.4	3.4	3.3	3.0
1/2 hr. after breakfast	3.7	2.9	3.1	5.3	4.5	3.5	5.9
1/2 hr. before lunch	3.9	3.0	3.1	4.9	4.3	4.7	3.6
1/2 hr. after lunch	3.7	3.1	3.6	4.8	3.4	4.4	3.1
1/2 hr. before supper	3.2	2.9	3.8	4.8	5.1	3.4	3.1
1/2 hr. after supper	3.5	2.8	3.7	5.3	3.7	3.7	3.4
Before retiring	4.0	2.9	3.5	3.9	lost	4.8	3.3

	L	T	K	Th	M	E	Lo	H
					Men graduate students			
1/2 hr. before breakfast	4.9	3.0	4.8	3.2	5.3	3.5	3.9	4.7
1/2 hr. after breakfast	5.0	3.1	5.4	3.2	5.3	4.9	3.6	5.0
1/2 hr. before lunch	4.6	3.5	5.2	3.2	5.7	4.4	4.4	4.6
1/2 hr. after lunch	4.6	3.6	4.8	3.5	5.6	4.6	4.5	5.5
1/2 hr. before supper	4.9	3.4	4.4	3.5	5.8	4.7	4.5	5.2
1/2 hr. after supper	4.9	3.9	5.4	3.2	5.8	4.9	4.6	5.1
Before retiring	4.8	3.8	5.6	3.1	5.6	4.0	3.8	4.6

all rats showed deposits. Large patches of deposit were found on the teeth of the animals that had consumed 130 mg of oxalate during a period of 13 days. These deposits were usually much more marked on the upper molars than on the lower ones.

Various trials were made to determine if sodium oxalate dissolved in drinking water would produce a film that might afford protection at a later date against acid beverages. Fif-

teen milligrams of sodium oxalate consumed daily for 25 days afforded no protection when rats were subsequently given 15 ml of cola beverage daily for 4 to 7 days. Even the feeding of 10 ml of 0.1% sodium oxalate dissolved in a 10% sucrose solution in the morning, followed by a cola beverage solution 5 hours later in the afternoon, afforded little protection against acid erosion.

No method was discovered of using oxalate to prevent acid erosion which proved superior to that described in an earlier report (Gortner et al., '46). Earlier experiments were repeated by scoring the teeth of rats given 10 ml of a cola beverage daily for 28 days. Nine rats received this beverage in this amount daily, while another comparable 9 were given the beverage containing 0.1% of sodium oxalate. The average total erosion score was 16 for the rats receiving oxalate compared with 46 for the animals given the acid beverage without oxalate; these scores indicate the degree of erosion.

Rhubarb and lemon juice

Since rhubarb juice has long been used as a beverage, and since it affords a natural source of oxalate that will counteract the acid erosion of lemon juice when the two are combined, trials were run to determine the useful combinations. Rhubarb juice was extracted and canned after the stalks were steamed in a pressure cooker. The leaves of the rhubarb were not included, although they are known to be rich in oxalic acid. All solutions tested were sweetened by the presence of 20% sucrose.

The test consisted of allowing individual rats 15 ml of the combined juices daily for 14 days. After this the rats were killed and the teeth scored in the usual manner. The ratios tested were rhubarb: lemon, 1:1, 1:4, and 1:9.

The results of these tests indicated that the ratio 1:1 afforded almost complete protection at the base of the teeth but allowed some erosion at the tips. The other ratios afforded no protection. No studies were made to determine whether

this protective effect of rhubarb juice was due solely to its content of oxalate.

If these combinations of juices were diluted as would be done for human consumption, the ratio of the rhubarb juice had to be increased.

Phytate and stearates as protective agents

Since both phytic and stearic acids form insoluble calcium compounds, it seemed possible that these might provide protective films on teeth. Calcium phytate, to make a solution of 0.03%, was dissolved in a phosphoric acid-sucrose solution similar to a cola beverage. Two groups of rats containing 5 animals each were fed this solution in the amount of 20 ml per day for 8 and 14 days, respectively. At the end of these periods the rats were killed and the erosion of their teeth compared with that of animals fed the solution without the phytate. No evidence was found of protective action by the phytate.

Calcium phytate was also fed mixed with a meal so that each rat consumed 20 mg daily for 14 days. These rats were allowed to drink 20 ml of cola beverage on the 15th day. Controls not given the phytate but drinking the same amount of acid beverage were used. No evidence was found of film formation or protection by phytate under these conditions.

Stearic acid was tested using 8 groups of 5 rats each. The acid was mixed into the meal diet for 6 of these groups at the following levels: 50, 100, 200, 400, 800, and 1,600 mg%. Two groups were used for purposes of comparison; one was fed the basal diet alone and the other the basal diet mixed with 50 mg% of sodium oxalate.

Each rat was allowed 20 ml daily for two weeks of the usual phosphoric acid-sucrose solution. Examination of the teeth of these animals at the end of this period revealed the usual deposit from the sodium oxalate but no film nor protection in any rat fed the different levels of stearic acid.

These exploratory studies afford no evidence that either phytic or stearic acid will develop protective films against acid erosion.

The feeding of sodium oxalate to beef steers

In the hope of getting enough of the deposit that forms on the teeth of animals fed sodium oxalate for analysis, the powdered oxalate was fed to 4 beef steers being fattened for slaughter. Each was fed 7 gm daily of powdered sodium oxalate mixed with the concentrate. This was continued for one month. After this period the steers were slaughtered and the jaws removed.

No evidence of any deposit was found, indicating either that conditions in the mouth prevented deposition or that some action such as rumination removed the deposit.

Toxicity of sodium oxalate to dogs

Although the early literature (Kobert and Kussner, 1879) gave substantial attention to the toxicity of oxalic acid, modern research has devoted scant consideration to the subject. In the studies of Hammersten ('37), dietary oxalate seemed to play a minor role in the production of oxalate stones in rats. Other dietary factors such as magnesium and the fat soluble vitamins appeared to be more important.

In order to determine whether injury could be detected after feeding oxalate to animals daily for a substantial period, a study covering 15 months was begun. Five young, adult mongrel dogs weighing 26 to 36 lb. were fed 100 mg of powdered oxalate daily, mixed with the stock diet. Post mortem examination yielded no evidence of stone formation or pathological changes. These dogs were maintained on the same complete stock diet which was fed to the rats in the erosion studies.

SUMMARY

In the case of the rat, erosion of the molar teeth can be detected after the consumption of 10 ml of acid solutions of the

same strength used in cola beverages. A comparative test of acid erosion during a period of 6 months indicated that tomato juice had the least effect and 0.055% phosphoric acid the most. Rat teeth subjected to phosphoric acid for this period were eroded nearly to the gum line and erosion was too severe for scoring in the conventional manner.

Human teeth suspended in cola beverage or its equivalent in sucrose-phosphoric acid gradually lose calcium in the course of two weeks.

The buffer capacity of the human mouth against cola beverages differs widely but is fairly consistent for a given individual. Even after a half minute's exposure some mouths cannot buffer the solution to a pH higher than 3.5, which is considered marginal for erosion.

A series of tests indicates that sodium oxalate ingested with food deposits patches on the teeth but is not as effective in the prevention of erosion as oxalic acid dissolved in an acid beverage. Rhubarb juice combined with equal parts of lemon juice protects the teeth against erosion.

Neither stearic nor phytic acids prevented erosion by acid beverages.

Oxalate patches could not be deposited on the teeth of steers during a period of one month's feeding of sodium oxalate.

No evidence of injury or stone formation was found after feeding adult dogs 100 mg of sodium oxalate daily for 15 months.

LITERATURE CITED

BIERI, J. G., C. M. MCCAY, J. S. RESTARSKI AND R. A. GORTNER, JR. 1946 Further studies on *in vivo* tooth decalcification by acid beverages. Arch. Biochem., *11:* 33.

GORTNER, R. A., JR., C. M. MCCAY, J. S. RESTARSKI AND C. A. SCHLACK 1946 Some effects of dietary oxalate on the teeth of white rats. J. Nutrition, *32:* 121.

HAMMERSTEN, G. 1937 Calciumoxalate als Steinbilder in den Harnwegan. Lunds Universitets Arsskrift, Avd. 2, Bd. 32, Nr. 12, 1–155.

KOBERT, R., AND B. KUSSNER 1879 Die experimentellen Wirkungen der Oxalsaure. Arch. Path. Anat., 78–209.

MCCLELLAND, JOHN R. 1926 The decalcification of human tooth enamel. Dent. Cosmos, *68:* 127.

McCLURE, F. J. 1943 The destructive action *in vivo* of dilute acids and acid drinks and beverages on the rats' molar teeth. J. Nutrition, *26:* 251.

PICKERILL, H. P. 1912 The Prevention of Dental Caries and Oral Sepsis. Baillière, Tindall and Cox, London, p. 140.

RESTARSKI, J. S., R. A. GORTNER, JR. AND C. M. McCAY 1945 A method for measuring the effects of acid beverages on the teeth of small laboratory animals. Science, *102:* 404.

SMITH, C. A. H. 1946 Hard tissue lesions of the mouth. J. Am. Dent. Assoc., *33:* 214.

STAFNE, E. C., AND S. A. LOVESTEDT 1947 Dissolution of tooth substance by lemon juice, acid beverages and acids from other sources. Ibid., *34:* 586.

WYNN, W., AND J. HALDI 1948 The erosive action of various fruit juices on the lower molar teeth of the albino rat. J. Nutrition, *35:* 489.

THE EFFECT OF SUPPLEMENTAL METHIONINE ON THE NUTRITIVE VALUE OF DIETS CONTAINING CONCENTRATES OF THE SOYBEAN TRYPSIN INHIBITOR [1]

I. E. LIENER, H. J. DEUEL, JR. AND H. L. FEVOLD

Department of Biochemistry and Nutrition, School of Medicine, University of Southern California, Los Angeles,[2] *and the Food Research Division, Quartermaster Food and Container Institute for the Armed Forces, Chicago, Illinois* [3]

(Received for publication June 13, 1949)

The marked improvement which proper heat treatment exerts on the nutritive value of soybean protein has been observed by numerous investigators, including Osborne and Mendel ('17); Hayward, Steenbock and Bohstedt ('36); Wilgus, Norris and Heuser ('36); Fritz, Kramke and Reed ('47); Klose, Hill and Fevold ('48); Borchers, Ackerson and Mussehl ('48a), and others. It has been further established that the supplementation of raw soybean meal with methionine is an effective means of largely eliminating the marked discrepancy in nutritive value between raw and properly heated soybean meal (Hayward and Hafner, '41; Evans and McGin-

[1] This investigation, undertaken in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Southern California by one of the present authors, was conducted at the Quartermaster Food and Container Institute for the Armed Forces, Chicago, Illinois.

[2] Contribution number 222 from the Department of Biochemistry and Nutrition, University of Southern California, Los Angeles.

[3] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 264 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

nis, '46; Gerry, Carrick and Hauge, '48). The effectiveness of supplemental methionine in this respect has been generally attributed to the increased availability of the sulfur-containing amino acids in the heated soybean protein. Johnson, Parsons and Steenbock ('39) demonstrated in the rat that this difference in the availability of the sulfur-containing amino acids between raw and heat-processed soybean meals could not be explained by any difference in the amount of sulfur absorbed from the intestine. Based on their own observation that the effect of heat on soybean protein was to increase the rate of release of methionine to a greater extent than that of leucine and lysine, Melnick, Oser and Weiss ('46) have suggested that, in the case of raw soybean, methionine is released too slowly in the intestinal tract to be available for simultaneous utilization with the other essential amino acids.

Attention has recently been directed to the presence of one or more antitryptic factors in unheated soybean meal (Bowman, '44, '46, '48; Ham and Sandstedt, '44; Kunitz, '46, '47) and to the deleterious effect of trypsin inhibitor preparations on the growth of experimental animals (Ham, Sandstedt and Mussehl, '45; Klose, Hill, and Fevold, '46a; Borchers, Ackerson, and Mussehl, '48b). Westfall and Hauge ('48) were able to correlate the improvement in the nutritive value of soybean protein effected by heat treatment with destruction of trypsin inhibitor potency. Although these lines of investigation suggest that the poor growth-promoting value of raw soybean meal is due to an inhibition of proteolytic digestion in the intestine, affecting the enzymatic release of methionine, recently reported experiments by Desikachar and De ('47), Klose, Greaves and Fevold ('48), and Westfall, Bosshardt and Barnes ('48) have demonstrated the growth-depressing effect of active antitryptic preparations even when incorporated into diets containing pre-digested protein.

The present investigation was undertaken in an attempt to clarify the mechanism of this anomalous behavior of the soy-

bean trypsin inhibitor, especially in relation to the well-known effectiveness of methionine as a supplement to raw soybean meal.

EXPERIMENTAL

A trypsin inhibitor concentrate was prepared from solvent-extracted soybean flour [4] according to the procedure of Klose, Hill and Fevold ('46a). The antitryptic activity of this preparation was found to be approximately one-third that of the crystalline soybean trypsin inhibitor.[5]

The soybean hydrolysate was prepared by autoclaving solvent-extracted soybean flour with 4 parts of 9 N sulfuric acid at 15 lb. pressure for 18 hours. The sulfuric acid was removed with barium hydroxide to pH 4.2, and the resultant filtrate was concentrated *in vacuo* and lyophilized.

The autoclaved soybean meal used in this study was obtained by subjecting the solvent-extracted soybean flour to 15 lb. pressure for 20 min. (115°C.) while spread in the bottom of an enameled pan to a depth of about one inch. The autoclaved material was finely comminuted before being mixed into the appropriate diet.

The composition of the 4 basal diets employed in this study is presented in table 1. A protein level of 12% (N × 6.25) was chosen for the diets containing soybean protein because it has been shown that this level gives the maximum utilization of nitrogen for growth (Barnes, Maack, Knights and Burr, '45; Westfall and Hauge, '48). In the case of the hydrolysate, DL-tryptophan and L-cystine were added to compensate for possible losses incurred during acid hydrolysis; the amounts of each required to restore the original level of these amino acids in the unhydrolyzed protein were added. Also included in this study were diets containing casein provided at a level of 18% protein. The trypsin inhibitor preparation and DL-methionine, when used as adjuncts to the basal diets, were added without an equivalent deduction in protein in order

[4] Nutrisoy XXX, Archer-Daniels-Midland, Minneapolis, Minn.

[5] Generously provided by Dr. M. Kunitz, The Rockefeller Institute for Medical Research, Princeton, New Jersey.

to eliminate the possibility that any inhibiting effect would be even partly due to a lowered concentration of available amino acids in the diet.

Fifteen groups of 7 weanling male rats each (Sprague-Dawley) were placed on the various experimental diets when

TABLE 1

Composition of basal diets used to demonstrate the effect of supplemental methionine in the presence of the soybean trypsin inhibitor

INGREDIENT	DIET NUMBER			
	A	B	C	D
	%	%	%	%
Basic mix [1]	20.00	20.00	20.00	20.00
Nutrisoy XXX [2]	23.40			
Autoclaved Nutrisoy XXX [3]		23.85		
Soybean hydrolysate [4]			18.25	
L-cystine [5]			0.25	
DL-tryptophan [6]			0.15	
Casein, vitamin-free [7]				20.25
Corn starch	56.60	56.15	61.35	59.75

[1] Provides the following components per 100 gm diet: Wesson oil, 5.0 gm; salt mix, U.S.P. 2, 4.0 gm; sucrose, 9.0 gm; Wilson's 1–20 liver powder, 0.4 gm; riboflavin, 1.0 mg; thiamine, 0.5 mg; pyridoxine, 0.5 mg; niacin, 2.0 mg; calcium pantothenate, 2.5 mg; para-aminobenzoic acid, 25.0 mg; choline, 200 mg; inositol, 20.0 mg; 2-methyl-1, 4-naphthoquinone, 1.0 mg; biotin, 0.02 mg; alpha-tocopherol, 2.5 mg; haliver oil, 50 mg.

[2] Solvent-extracted soybean flour, equivalent to 12% protein based on N value of 8.21%.

[3] Equivalent to 12% protein based on N value of 8.05%.

[4] Equivalent to 11.7% protein based on N value of 10.25%.

[5] Equivalent to 0.18% protein and calculated to give a cystine value of 1.9 gm/16 gm N reported for soybean protein (Block and Mitchell, '46).

[6] Equivalent to 0.13% protein and calculated to give a tryptophan value of 1.2 gm/16 gm N reported for soybean protein (Block and Mitchell, '46).

[7] Equivalent to 18% protein based on N value of 14.21%.

they weighed 35 to 45 gm. The animals were put in individual cages provided with wire mesh bottoms. Food and water were provided ad libitum for a period of 16 days, during which time daily records were kept of individual food consumption and weight. The spillage of food was minimized by using small ointment jars placed in larger containers so that the whole

feeding apparatus could be weighed together, thus avoiding any loss of the small amounts of food spilled into the outer container. As a further precaution, sheets of paper were placed under each cage so that excessive amounts of spilled food could always be recovered.

The protein efficiency ratio (Osborne, Mendel and Ferry, '19), calculated as the grams gain in weight per gram of protein consumed, was employed as an index of the growth-promoting value of the various experimental diets.[6] These results are given in table 2. Apparent differences were evaluated by the "t" test (Snedecor, '46) as is shown in table 3, and only those showing a P value of less than 0.01 were considered significant. The reliability of protein-efficiency ratios determined over relatively short feeding periods has been pointed out by Klose, Greaves and Fevold ('48) and by Russell, Taylor, Mehrof and Hirsch ('46).

RESULTS

It will be noted in table 2 that supplemental methionine improved the nutritive value of all diets, including those from which the trypsin inhibitor had been omitted. Thus any effect which supplemental methionine may have in conjunction with the trypsin inhibitor is obscured. In order to differentiate quantitatively between these two effects, the percentage increase in protein efficiency (PE) over that of the unsupplemented diets as a consequence of the addition of methionine was calculated. The relative effectiveness of supplemental methionine upon the growth-promoting value of the various diets could then be judged by the magnitude of the differences in percentage units.

[6] The authors recognize the limitations of this method of evaluating the nutritive value of dietary protein, which Mitchell ('44) has criticized on various grounds. The work of Barnes, Maack, Knights and Burr ('45), however, would appear to justify the use of the maximum PE ratio as a reliable index of the relative growth-promoting quality of dietary protein. Since much of the previous work relating to growth inhibitors derived from soybean has involved measurement of the gain in weight in relation to protein consumption, choice of this procedure by the authors was further governed by the desire to obtain data which would permit comparison with reported results in the literature.

It becomes apparent that methionine added to the raw soybean meal diet (compare groups 1 and 2) or to the autoclaved soybean meal diet containing the trypsin inhibitor (compare groups 5 and 6) was relatively more effective in promoting growth than when added to autoclaved soybean meal alone (compare groups 3 and 4). In the latter case, its relative in-

TABLE 2

The effect of supplemental methionine on the nutritive value of diets containing a crude concentrate of the soybean trypsin inhibitor

GROUP	DIET	PROTEIN [1]	AVG. GAIN IN WEIGHT	AVG. FOOD CONSUMPTION	AVG. PROTEIN CONSUMPTION	PROTEIN EFFICIENCY [2]	EFFECTIVENESS OF SUPPLEMENTAL METHIONINE [3]
		%	gm	gm	gm		%
1	Raw soybean	12.7	19.1	107.2	13.6	1.40 ± 0.15	
2	Raw soybean + 0.6% methionine	13.1	46.4	146.4	19.2	2.42 ± 0.10	74.0
3	Autoclaved soybean	12.5	61.6	187.0	23.4	2.63 ± 0.10	
4	Autoclaved soybean + 0.6% methionine	14.1	82.6	196.1	27.6	2.99 ± 0.03	13.7
5	Autoclaved soybean + 1.8% inhibitor	13.8	37.3	138.5	19.1	1.95 ± 0.09	
6	Autoclaved soybean + 1.8% inhibitor + 0.6% methionine	14.6	61.8	161.0	23.5	2.63 ± 0.07	34.8
7	Soybean hydrolysate	13.1	25.7	110.9	14.5	1.77 ± 0.06	
8	Soybean hydrolysate + 0.6% methionine	13.0	26.3	98.1	12.7	2.07 ± 0.05	16.9
9	Soybean hydrolysate +1.8% inhibitor	13.6	21.6	103.0	14.0	1.54 ± 0.03	
10	Soybean hydrolysate + 1.8% inhibitor + 0.6% methionine	14.1	25.4	100.0	14.1	1.80 ± 0.04	16.8
11	Soybean hydrolysate + 1.8% autoclaved inhibitor [4]	13.7	23.7	94.5	12.9	1.84 ± 0.05	
12	18% casein	19.0	90.6	176.5	33.6	2.69 ± 0.06	
13	18% casein + 0.6% methionine	19.3	92.3	158.5	30.6	3.01 ± 0.04	11.9
14	18% casein + 1.8% inhibitor	20.2	76.7	163.3	33.0	2.32 ± 0.05	
15	18% casein + 1.8% inhibitor + 0.6% methionine	20.6	90.3	167.4	34.5	2.61 ± 0.08	12.5

[1] N × 6.25.

[2] Protein efficiency (grams gain in weight per gram protein consumed) determined over a feeding period of 16 days. The mean of each group (7 animals) ± the standard error of the mean.

[3] The per cent increase in protein efficiency when diet is supplemented with DL-methionine, calculated as follows:

$$\frac{PE(\text{diet} + \text{methionine}) - PE(\text{diet} - \text{methionine})}{PE(\text{diet} - \text{methionine})} \times 100.$$

[4] Autoclaved at 15 lb. for 20 min.

effectiveness can be adequately explained by the fact that methionine is the limiting amino acid of soybean protein even when properly heated (Almquist, Mecchi, Kratzer and Grau, '42). The greater efficacy of methionine with raw soybean or autoclaved soybean plus inhibitor suggests that a factor other than a deficiency of methionine is involved, which interferes

TABLE 3

Statistical evaluation of protein efficiency ratios by the "t" test[1]

GROUP NUMBER	COMPARED WITH GROUP NUMBER				
	Basal protein: Raw or autoclaved soybean protein				
	2	3	4	5	6
1	5.80	7.05	16.75	3.46	7.24
2		1.58	5.82	3.34	1.88
3			3.66	4.96	0.08
4				11.23	4.85
5					5.65
	Basal protein: Soybean hydrolysate				
	8	9	10	11	
7	3.56	6.48	1.26	0.78	
8		12.65	6.18	3.29	
9			4.06	7.81	
10				2.57	
	Basal protein: "18%" casein				
		13	14	15	
12		3.85	4.15	0.65	
13			9.65	4.05	
14				2.90	

[1] Calculated according to Snedecor ('46). Only those "t" values greater than 3.055 ($P < .01$) were considered indicative of significant differences.

with the availability of methionine for growth. This is not the whole explanation, however. If the only effect of the inhibitor were to decrease the availability of methionine, then adding an excess of methionine to either soybean (group 2) or to autoclaved soybean plus inhibitor (group 6) should elevate their PE to the same level as that of methionine-supplemented autoclaved soybean (group 4). This disparity, which persists in spite of the adequacy of methionine, is indicative

of still another factor, not involving the availability of methionine, which adversely affects growth.

The lower PE values obtained for the diets containing the soybean hydrolysate may be attributed to the inferiority of hydrolyzed soybean protein to unhydrolyzed protein in respect to maintenance of a positive N balance which was observed by Supplee and Clark ('46). In contrast to the results observed with the intact soybean protein, the improvement in the nutritive value of the diet containing soybean hydrolysate and inhibitor as a result of supplementation with methionine (compare groups 9 and 10) was not appreciably different from the effect produced by adding methionine to the hydrolysate alone (compare groups 7 and 8). In this instance, therefore, the beneficial effect of methionine could be essentially accounted for by the fact that the hydrolysate was deficient in this amino acid. The difference in PE between the diet containing methionine-supplemented hydrolysate (group 8) and methionine-supplemented hydrolysate plus inhibitor (group 10) is again attributable to a mechanism totally unrelated to the utilization of methionine for growth. The additional protein furnished by the trypsin inhibitor preparation did not significantly affect the PE of the basal diet (compare groups 7 and 11).

It was thought that the incorporation of casein at an 18% protein level would provide a diet in which the effectiveness of methionine in relation to the inhibitor could be studied directly, without the complication of a methionine deficiency. The data in table 2, however, show that the 18% casein diet could be further supplemented with methionine. Subsequent examination of the literature revealed that at least one paper has reported the same observation (Mulford and Griffith, '42). Data relating to the relative effectiveness of supplemental methionine are similar to the results obtained with the soybean hydrolysate. As in the case of the latter, the effect of supplemental methionine on the diets containing casein and

the inhibitor (compare groups 14 and 15) [7] was about the same as would be expected to result from the addition of methionine to diets containing casein alone (compare groups 12 and 13). Consistent with the data obtained with intact and hydrolyzed soybean protein is the observation that the diet containing 18% casein plus inhibitor (group 15) remained nutritionally inferior to a similar diet from which the inhibitor had been omitted (group 13) even when both diets were adequately supplemented with methionine (see table 3, last column, "t" = 4.05).

DISCUSSION

On the basis of the foregoing evidence, it becomes necessary to postulate that the mode of action of concentrates of the trypsin inhibitor with respect to growth may involve two mechanisms: (A) one which interferes with the availability of methionine for growth; and (B) one which exerts a growth-depressing effect through a mechanism unrelated to A. When the source of N in the diet is intact soybean protein, it appears that both mechanisms A and B are responsible for growth inhibition; when the N is contributed by hydrolyzed soybean protein, mechanism B is apparently the only causative factor involved. Since mechanism A did not come into play when the protein had been subjected to complete hydrolysis, it is probable that this mechanism depends on the antiproteolytic action of the inhibitor. Similarly, mechanism B does not depend on an inhibition of proteolysis. Since a crude concentrate of the trypsin inhibitor was used in this study, it cannot be inferred that mechanisms A and B are necessarily caused by the same substances.

The demonstration by Hayward and Hafner ('41), Almquist, Mecchi, Kratzer and Grau ('42), and others that the addition of methionine to raw soybean meal improves growth to a greater extent than when it is added to autoclaved soybean meal has been interpreted to indicate that heating in-

[7] The effectiveness of supplemental methionine in this instance was not quite significant at the 1% level. See table 3.

creases the availability of methionine from soy protein. The data presented here substantiate this view and suggest further that it is the trypsin inhibitor of unheated soybean meal which interferes with the availability of methionine for growth. It has, however, never been entirely clear why the nutritive value of raw and autoclaved soybean meal should not be equalized when both are supplemented with an excess of methionine. Hayward and Hafner ('41) have suggested that autoclaving soybean meal raises its general plane of nutrition by increasing the availability of other amino acids as well, which might have been the limiting factor for growth in the case of the unheated meal even in the presence of adequate amounts of supplemental methionine. The absence of other limiting amino acids in raw soybean meal, however, has been demonstrated by Klose, Hill and Fevold ('46b). These workers showed that methionine alone was just as effective a supplement to raw soybean meal as all 10 essential amino acids. It appears quite likely, therefore, that this discrepancy between raw and autoclaved soybean meal when both are supplemented with methionine can be more simply explained by the hypothesis that the trypsin inhibitor (or some associated substance) exerts a separate growth-retarding effect, designated herein as mechanism B. This same effect could very well account for the ability of active trypsin inhibitor preparations to inhibit the growth of animals receiving diets containing protein hydrolysates.

Although the crude trypsin inhibitor preparation depressed the growth of rats receiving a diet containing 18% casein, this inhibition did not appear to involve any interference with the availability of methionine for growth, as judged by the effectiveness of supplemental methionine. If mechanism B, therefore, is the only factor responsible for the adverse effect of the trypsin inhibitor on the nutritive value of diets containing casein, it must follow that the inhibitor will (1) have the same quantitative effect on either casein or its hydrolysate, and (2) retard growth to a greater extent when added to diets containing properly heated soybean protein, where

both mechanisms A and B operate, then when incorporated into diets containing casein. Positive evidence for both of these points is at hand. With respect to (1), Klose, Greaves and Fevold ('48) and Westfall, Bosshardt, and Barnes ('48) found that trypsin inhibitor preparations depressed the growth of rats on diets containing casein hydrolysates to at least the same extent as they did the growth of those fed unhydrolyzed casein. Regarding (2), Westfall and Hauge ('48) found that the deleterious effect of the inhibitor on the nutritive value of casein was less than on properly heated soybean flour. Similarly, Ham, Sandstedt and Mussehl ('45) observed that a trypsin inhibitor prepared by acetone precipitation from aqueous extracts of raw soybean exerted a greater inhibitory effect on the growth of chicks fed autoclaved soybean meal than on the growth of those fed protein of animal origin.

Recent papers (Riesen, Clandinin, Elvehjem and Cravens, '47; Hou, Riesen and Elvehjem, '49; Liener and Fevold, '49) have shown that the rate of release of methionine remains an inadequate explanation for the poor nutritive value of raw soybean. Nevertheless, the observation that the interference with the availability of methionine exerted by the trypsin inhibitor depends on its antiproteolytic activity invites a reevaluation of the concept relating to inhibition of the rate of release of methionine (Melnick, Oser and Weiss, '46). If one considers the rate of intestinal absorption of the amino acids (Wilson and Lewis, '29; Chase and Lewis, '33) as well as their rate of liberation, the mutual supplementation of these acids could be expected to play a significant role in their utilization by the animal organism, in the following manner. If, in unheated soybean meal, the rate of proteolytic digestion is lower than the absorptive capacity of the intestine, there would be no tendency for amino acids to accumulate in the intestinal tract, and the concentration of the limiting amino acid, methionine, may be insufficient to permit adequate supplementation of the other amino acids. On the other hand, if, after the meal has been heated to inactivate the trypsin

inhibitor, the rate of digestion is higher than the intestinal absorptive capacity, the digestion products would tend to accumulate, and the methionine concentration may be great enough to act in a mutually supplementary manner with the other amino acids which are in excess. In the case of proteins of animal origin such as casein, in which methionine is relatively more abundant,[8] it is possible that the differential between the rate of proteolytic digestion, as affected by the trypsin inhibitor, and the rate of intestinal absorption may not be great enough to depress the concentration of methionine to the point where it will not adequately supplement the other amino acids. Under such conditions, the ability of the trypsin inhibitor to interfere with the availability of methionine would not play a significant role in the over-all effect of the inhibitor on the nutritive value of casein and other proteins in which a deficiency of methionine is not as critical as in the case of soybean protein.

SUMMARY

Animal experiments involving measurement of the growth performance of rats were conducted to determine the effect of supplemental methionine on diets containing a soybean trypsin inhibitor concentrate and in which the sources of nitrogen were raw or autoclaved soybean protein, soybean protein hydrolysate, or casein. When the soybean hydrolysate or casein was furnished in the diet, the depression in growth exerted by the trypsin inhibitor preparation was not related to interference with the availability of methionine for growth. The beneficial effect of methionine in this instance could be wholly accounted for by the fact that the hydrolysate and casein were deficient to some extent in this amino acid. With diets containing autoclaved soybean meal, the growth-retarding action of the inhibitor was referable to interference with the availability of methionine for growth, as well as to the deficiency observed in the case of the hydrolysate and casein.

[8] Block and Mitchell ('46) report the methionine content of casein and soybean protein to be 3.5 and 2.0 gm/16 gm N, respectively.

These results may be explained by postulating a two-fold mode of action by active trypsin inhibitor preparations, one which results in an impairment of the availability of methionine for growth as a consequence of its antiproteolytic activity, and the other having an effect unrelated to the inhibition of proteolysis. The former can be demonstrated if the source of ingested N is an intact protein critically deficient in methionine. The latter effect is independent of the nature and quality of ingested N.

LITERATURE CITED

ALMQUIST, H. J., E. MECCHI, F. H. KRATZER AND C. R. GRAU 1942 Soybean protein as a source of amino acids for the chick. J. Nutrition, *24:* 385.

BARNES, R. H., J. E. MAACK, M. J. KNIGHTS AND G. O. BURR 1945 Measurement of the growth promoting quality of dietary protein. Cereal Chem., *22:* 273.

BLOCK, R. J., AND H. H. MITCHELL 1946 The correlation of the amino acid composition of proteins with the nutritive value. Nutrition Abstr. and Rev., *16:* 249.

BORCHERS, R., C. W. ACKERSON AND F. E. MUSSEHL 1948a Trypsin inhibitor. VI. Effect of various heating periods on the growth promoting value of soybean oil meal for chicks. Poultry Sci., *27:* 601.

———— 1948b Trypsin inhibitor. VIII. Growth inhibiting properties of a soybean trypsin inhibitor. Arch. Biochem., *19:* 317.

BOWMAN, D. E. 1944 Fractions derived from soybeans and navy beans which retard the tryptic digestion of casein. Proc. Soc. Exp. Biol. Med., *57:* 139.

———— 1946 Differentiation of soybean antitryptic factors. Ibid., *63:* 1946.

———— 1948 Further differentiation of bean trypsin inhibiting factors. Arch. Biochem., *16:* 109.

CHASE, B. W., AND H. B. LEWIS 1933 The metabolism of sulfur. The rate of absorption of dl-methionine from the gastrointestinal tract of the white rat. J. Biol. Chem., *101:* 735.

DESIKACHAR, H. S. R., AND S. S. DE 1947 Role of inhibitors in soybean. Science, *106:* 421.

EVANS, R. J., AND J. MCGINNIS 1946 The influence of autoclaving soybean oil meal on the availability of cystine and methionine for the chick. J. Nutrition, *31:* 449.

FRITZ, J. C., E. H. KRAMKE AND C. A. REED 1947 Effect of heat treatment on the biological value of soybeans. Poultry Sci., *26:* 657.

GERRY, R. W., C. W. CARRICK AND S. M. HAUGE 1948 Untoasted soybean oil meal in a simplified chick ration. Ibid., *27:* 621.

HAM, W. E., AND R. M. SANDSTEDT 1944 A proteolytic inhibiting substance in the extract from unheated soybean meal. J. Biol. Chem., *154:* 505.

HAM, W. E., R. M. SANDSTEDT AND F. E. MUSSEHL 1945 The proteolytic inhibiting substance in the extract from unheated soybean meal and its effect upon growth in chicks. Ibid., *161:* 635.

HAYWARD, J. W., AND F. H. HAFNER 1941 The supplementary effect of cystine and methionine upon the protein of raw and cooked soybeans as determined with chicks and rats. Poultry Sci., *20:* 139.

HAYWARD, J. W., H. STEENBOCK AND G. BOHSTEDT 1936 The effect of heat as used in the extraction of soybean oil upon the nutritive value of the protein of soybean oil meal. J. Nutrition, *11:* 219.

HOU, H. C., W. H. RIESEN AND C. A. ELVEHJEM 1949 Influence of heating on the liberation of certain amino acids from whole soybeans. Proc. Soc. Exp. Biol. Med., *70:* 416.

JOHNSON, L. M., H. T. PARSONS AND H. STEENBOCK 1939 The effect of heat and solvents on the nutritive value of soybean protein. J. Nutrition, *18:* 423.

KLOSE, A. A., J. D. GREAVES AND H. L. FEVOLD 1948 Inadequacy of proteolytic inhibition as explanation for growth depression by lima bean protein fractions. Science, *108:* 88.

KLOSE, A. A., B. HILL AND H. L. FEVOLD 1946a Presence of a growth inhibiting substance in raw soybean. Proc. Soc. Exp. Biol. Med., *62:* 10.

———— 1946b Unpublished data.

———— 1948 Food value of soybean as related to processing. Food Technol., *2:* 201.

KUNITZ, M. 1946 Crystalline soybean trypsin inhibitor. J. Gen. Physiol., *29:* 149.

———— 1947 Crystalline soybean trypsin inhibitor. 2. General properties. Ibid., *30:* 291.

LIENER, I. E., AND H. L. FEVOLD 1949 The effect of the soybean trypsin inhibitor on the enzymatic release of amino acids from autoclaved soybean meal. Arch. Biochem., *21:* 395.

MELNICK, D., B. L. OSER AND S. WEISS 1946 Rate of enzymic digestion of proteins as a factor in nutrition. Science, *103:* 326.

MITCHELL, H. H. 1944 Determination of the nutritive value of the proteins of food products. Ind. Eng. Chem., Anal. Ed., *16:* 696.

MULFORD, D. J., AND W. H. GRIFFITH 1942 Choline metabolism. VIII. The relation of cystine and methionine to the requirement of choline in young rats. J. Nutrition, *23:* 91.

OSBORNE, T. B., AND L. B. MENDEL 1917 The use of soybean as food. J. Biol. Chem., *32:* 369.

OSBORNE, T. B., L. B. MENDEL AND E. L. FERRY 1919 A method of expressing numerically the growth promoting value of proteins. Ibid., *37:* 223.

RIESEN, W. H., D. R. CLANDININ, C. A. ELVEHJEM AND W. W. CRAVENS 1947 Liberation of essential amino acids from raw, properly heated, and overheated soybean oil meal. Ibid., *167:* 143.

RUSSELL, W. C., M. W. TAYLOR, T. G. MEHROF AND R. R. HIRSCH 1946 The nutritive value of the protein of varieties of legumes and the effect of methionine supplementation. J. Nutrition, *32:* 313.

SNEDECOR, G. W. 1946 Statistical Methods. The Iowa State College Press, Inc., Ames, Iowa.

SUPPLEE, G. C., AND E. L. CLARK 1946 Differential nitrogen retention from casein, lactalbumin, and soy protein and hydrolysates therefrom. J. Dairy Sci., *29:* 717.

WESTFALL, R. J., D. K. BOSSHARDT AND R. H. BARNES 1948 Influence of crude trypsin inhibitor on utilization of hydrolyzed protein. Proc. Soc. Exp. Biol. Med., *68:* 498.

WESTFALL, R. J., AND S. M. HAUGE 1948 The nutritive quality and the trypsin inhibitor content of soybean flour heated at various temperatures. J. Nutrition, *35:* 379.

WILGUS, H. S., JR., L. C. NORRIS AND G. F. HEUSER 1936 Effect of heat on nutritive value of soybean meal. Ind. Eng. Chem., *28:* 586.

WILSON, R. H., AND H. B. LEWIS 1929 Comparative studies on the metabolism of amino acids. II. The rate of absorption of amino acids from the gastro-intestinal tract of the white rat. J. Biol. Chem., *84:* 511.

THE EFFECT OF CARBOHYDRATE ON THE NUTRITIVE VALUE OF HEATED LACTALBUMIN [1]

IVAN J. MADER, LAWRENCE J. SCHROEDER AND ARTHUR H. SMITH
Department of Physiological Chemistry, Wayne University College of Medicine, Detroit, Michigan

THREE FIGURES

(Received for publication June 14, 1949)

It is generally accepted that the nutritive value of a protein depends essentially upon the kinds of amino acids which it contains and upon the quantitative proportion of each which is absorbed at a given time. Physical and chemical factors which destroy the amino acids or alter their availability to the organism will therefore affect the nutritional quality of the protein. Numerous investigations have been undertaken in an attempt to evaluate the effect of heat on proteins. These studies date from the work of McCollum and Davis ('15), who were probably the first to report that the protein constituent of milk is responsible for the loss in nutritive value which occurs when this fluid is heated. Fairbanks and Mitchell ('35) and Mumford ('33) also observed the sensitivity of milk proteins to heat. In addition, heating has been shown to produce detrimental effects on the nutritive value of beef protein (Morgan and Kern, '34; Seegers and Mattill, '35), fish meal (Schneider, '32; Maynard, Bender and McCay, '32), human globin (Devlin and Zittle, '44), field peas (Woods, Beeson and Bolin, '43), edestin (Waisman and Elvehjem, '38), pea-

[1] The data in this paper are taken from the dissertation submitted by Ivan J. Mader for the degree of Master of Science, Wayne University, 1949. A preliminary report of the work was given before the American Institute of Nutrition at Detroit, April 19, 1949.

nuts (Mitchell, Burroughs and Beadles, '36), cottonseed meal (Olcott and Fontaine, '41), gluten (Morgan and Petro, '30), soybean oil meal (Evans, McGinnis and St. John, '47), cereal proteins (Murlin, Nasset and Marsh, '38; Kuether and Myers, '48), and lactalbumin (Davis, Rizzo and Smith, '49).

Proteins and carbohydrates are known to interact under a variety of conditions. In 1912 Maillard described a reaction between amino acids and reducing sugars which formed humin-like substances, "melanoidins." This original observation was confirmed by Borsook and Wasteneys ('25), who reported a reduction of free amino nitrogen after an interaction between glucose and protein derivatives. The Maillard or melanoidin condensation theory is the classical explanation of non-enzymatic browning in food products (Stadtman, '48). The extent of the reaction appears to be in proportion to the availability of free aldehyde groups (Ransey, Tracy and Ruehe, '33; Ross, '48) and amino groups (Patton and Pyke, '46).

The effect of the browning reaction on the nutritive value of microbiological media has been investigated by Patton and Hill ('48), and Hill and Patton ('47), who reported the destruction of certain essential amino acids resulting from an interaction between the protein and reducing sugar components of the media. These results are in accord with those of Riesen et al. ('47), who studied the liberation of essential amino acids from heated soybean oil meal.

The widespread occurrence of the browning reaction and the suggestion that the interaction of carbohydrates and proteins is responsible for the nutritive damage which occurs when protein is heated, have prompted a further study of these effects in relation to the mammalian species. The present investigation has demonstrated the low nutritive value of heated protein for the adult dog and has described the effect of carbohydrate, when heated with the protein, on the nutritive value of the protein. The *in vivo* studies have been evaluated in terms of the reducing sugar content of the preparations used.

EXPERIMENTAL

Lactalbumin [2] was the protein used for all experiments. This protein will hereafter be referred to as "commercial lactalbumin." Preliminary analysis showed that it contained relatively large amounts of reducing substances which could be easily extracted with water. The following procedure was used to prepare the product hereinafter termed "extracted lactalbumin."

Two kilograms of commercial lactalbumin were suspended in 5 l of of distilled water and vigorously stirred mechanically for 30 min. The protein was recovered by centrifugation and resuspended in 5 l of water. This alternate washing and centrifugation operation was carried out 8 times over a period of three successive days. The protein was then washed in 5 l of 5% ethanol for 30 min., was centrifuged, washed finally with distilled water, again centrifuged, and allowed to dry in air. Thus, the commercial lactalbumin was extracted with 20 to 25 volumes of fluid. The extracted lactalbumin did not give a Molisch test.

Heat treatment of the commercial and extracted proteins included dry-heating or autoclaving, or both, for varying periods of time. For the dry-heating process, the lactalbumin was placed in shallow pans in layers not over 1 cm deep and baked in an oven at 120°C. for 60 min. When the protein was autoclaved it was placed in shallow pans and heated at 120°C. (15 lb. pressure) for 30 or 60 min. After autoclaving, the material was cooled rapidly, spread out in a thin layer and allowed to air-dry for at least three days before being used.

A purified ration containing all the known dietary essentials was prepared. The percentage composition of this basic diet, patterned after that used by Orten and Smith ('37) was: lactalbumin 10.0, sucrose 40.0, white dextrin 10.0, commercial hydrogenated cottonseed oil [3] 25.0, dried yeast [4] 6.8, Wesson salt mixture 3.5, bone ash 2.7, cod liver oil 2.0. The protein

[2] Lactalbumin 15–42, obtained from the Borden Company.

[3] Crisco.

[4] Anheuser-Busch, Strain G.

component of the diet was adjusted for all control and experimental periods to insure each animal's consumption of the same amount of nitrogen every day. Sucrose was correspondingly increased or decreased to make all rations isocaloric.

Three healthy adult female dogs, each weighing approximately 10 kg, were used as experimental subjects. They were kept in individual standard metabolism cages and had access to water at all times. The animals were fed at the same hour daily and weighed weekly. They ate their ration promptly and completely at all times.

The method employed for evaluating the nutritional quality of the proteins was adapted from the one used by Murlin and his associates ('41). This procedure involved a comparison of the amount of urinary and fecal nitrogen excreted by a subject fed a diet containing a "control" or reference protein with the amount excreted by the same subject when fed a similar diet containing the protein to be tested in place of the "control" protein. In the present study either commercial or extracted lactalbumin, unheated, served as the reference protein to which the heated proteins were compared. The experiment consisted of alternate control periods, each three days in length, and experimental periods of 4 days. The protein to be tested was fed for two 4-day periods separated by a control period.

Urine samples from each dog were obtained quantitatively by catheterization for all collection periods and were preserved with sulfuric acid and toluene. The urine was made up to volume and aliquots were analyzed for nitrogen. The fresh feces, separated into periods with carmine, were kept covered with ethanol containing 1% sulfuric acid until dried on a steam cone. Nitrogen determinations were made on aliquots of the ground dried material. All analyses for nitrogen were made in replicate by the macro-Kjeldahl method.

The caloric requirement of each dog was first determined with a diet containing 10% casein. That amount of ration which precluded any gain or loss in body weight was fed to the dog in all succeeding studies. A daily ration furnishing

45 to 75 cal. per kilogram of body weight was found to be satisfactory, depending upon the individual dog. The maximum weight change in any dog throughout the experiment was only 0.7 kg.

Before studying the effect of heat on the nutritive value of the lactalbumin it was desirable to conduct a number of balance studies which would determine the minimum amount of

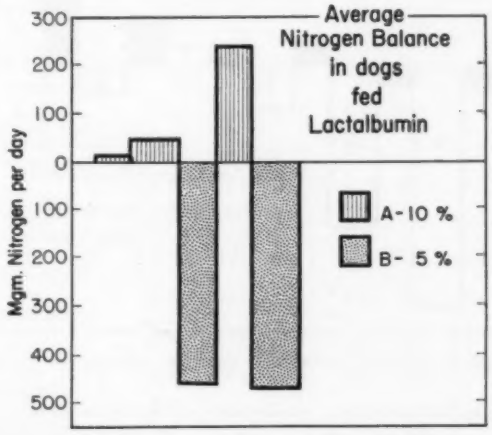


Fig. 1 Preliminary nitrogen balance studies to determine the minimum nitrogen requirement of the dogs.

protein necessary to keep the dogs in nitrogen balance. Accordingly, diets containing 5 and 10% of commercial lactalbumin were prepared and fed to the dogs in an amount commensurate with their caloric requirements. The averages of the nitrogen balances of the animals maintained on these diets are shown in figure 1. It is evident that lactalbumin fed at a level of 10% was adequate for nitrogen balance in the present study. The yeast of the diet supplied an appreciable, and constant, amount of nitrogen. This nitrogen comprised approximately 0.4% of the diet. There was a total dietary nitrogen content of about 1.67%, and all other diets contain-

ing a treated protein were constituted to contain as nearly as possible this amount of nitrogen.

Complete nitrogen balance data on each dog for all control and experimental periods are presented in table 1. Figures 2 and 3 show in chronological sequence the averages of the

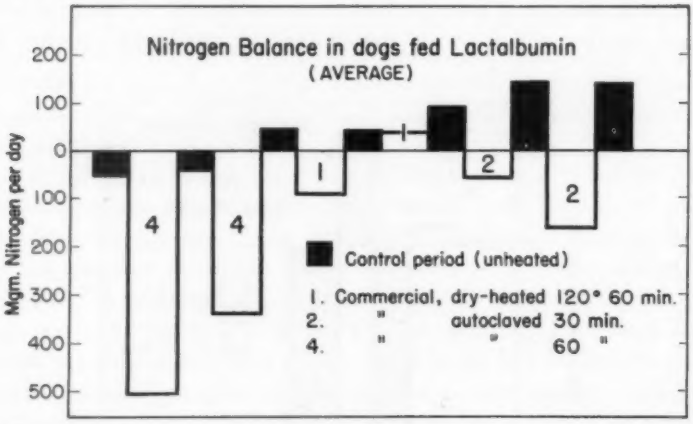


Fig. 2 Average nitrogen balances in dogs fed unextracted (commercial) lactalbumin.

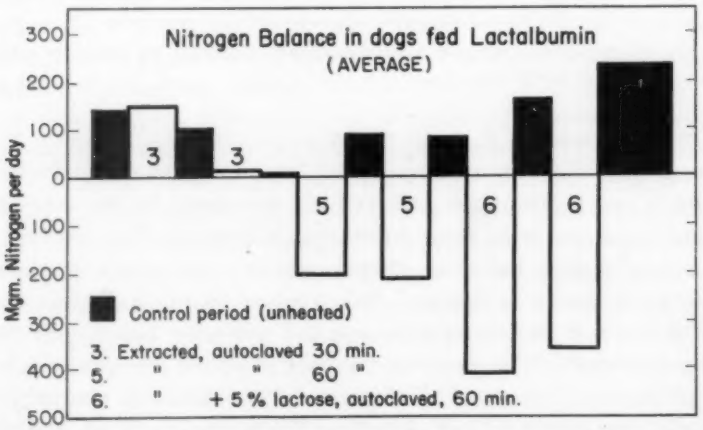


Fig. 3 Average nitrogen balances in dogs fed extracted (carbohydrate-free) lactalbumin.

TABLE 1

Food, urinary and fecal nitrogen data

	TOTAL FOOD N			TOTAL URINARY N			TOTAL FECAL N		
	Dog No.			Dog No.			Dog No.		
	1	2	3	1	2	3	1	2	3
	gm			gm			gm		
Commercial lactalbumin, unheated [1]	6.26	6.26	4.51	5.11	5.84	3.69	1.04	0.96	0.86
Commercial lactalbumin, autoclaved 60 min.[2]	8.15	8.15	5.25	6.16	6.83	4.78	4.03	2.98	2.87
Commercial lactalbumin, unheated	6.26	6.26	4.51	5.38	5.79	3.72	0.95	0.77	0.80
Commercial lactalbumin, autoclaved 60 min.	8.15	8.15	5.87	5.67	6.70	4.61	4.29	2.05	2.93
Commercial lactalbumin, unheated	6.26	6.26	4.51	4.55	5.74	3.44	1.05	0.96	0.86
Commercial lactalbumin, dry-heated 120°C. 60 min.	7.75	7.75	5.58	5.80	7.09	4.58	1.78	1.37	1.60
Commercial lactalbumin, unheated	6.34	6.34	4.56	4.89	5.72	3.51	1.01	0.87	0.86
Commercial lactalbumin, dry-heated 120°C. 60 min.	7.75	7.75	5.58	5.61	5.71	4.42	1.68	1.68	1.54
Commercial lactalbumin, unheated	6.38	6.38	4.59	4.78	5.12	3.67	1.04	1.02	0.88
Commercial lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	5.50	6.05	4.75	3.33	2.37	1.19
Commercial lactalbumin, unheated	6.38	6.38	4.59	4.47	5.21	3.46	1.08	1.03	0.84
Commercial lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	5.35	6.36	4.67	2.86	2.74	2.50
Commercial lactalbumin, unheated	6.38	6.38	4.59	4.58	5.21	3.49	1.07	0.84	0.92
Extracted lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	5.70	5.93	4.58	1.56	1.44	1.45
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.55	5.10	3.44	1.06	0.83	0.83
Extracted lactalbumin, autoclaved 30 min.	8.25	8.25	5.94	6.01	6.83	5.24	1.45	1.47	1.26
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.73	5.27	3.98	1.11	0.71	0.83
Extracted lactalbumin, autoclaved 60 min.	8.20	8.20	5.90	5.84	5.84	4.87	2.90	2.73	2.57
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.78	5.18	3.29	1.02	0.80	0.87
Extracted lactalbumin, autoclaved 60 min.	8.04	8.04	5.85	5.67	6.47	4.52	3.02	2.42	2.40
Extracted lactalbumin, unheated	6.15	6.15	4.43	4.55	5.22	3.41	0.96	0.93	0.91
Extracted lactalbumin + 5% lactose, autoclaved 60 min.	8.10	8.10	5.83	4.78	5.84	4.44	4.82	3.85	3.25
Extracted lactalbumin, unheated + 5% lactose	6.34	6.34	4.56	4.41	5.26	3.26	0.97	0.91	0.97
Extracted lactalbumin + 5% lactose, autoclaved 60 min.[3]	6.07	6.07	4.37	3.44	3.84	3.27	3.43	3.18	2.60
Extracted lactalbumin, unheated + 5% lactose[4]	12.67	12.67	9.13	7.53	9.97	6.44	2.27	2.30	1.74

[1] Each unheated protein was fed for a period of three days.
[2] Each heated protein was fed for a period of 4 days.
[3] Three-day period.
[4] Six-day period.

nitrogen balances of the three dogs as they were alternately fed the unheated and heat-treated proteins. Although there are individual variations in the extent of nitrogen balance, the direction of the change in nitrogen equilibrium for each dog is the same in all cases.

For calculating the digestibility and biological value of the heated proteins, a modification of the method proposed by Murlin et al. ('41) was used. It was assumed that the absorption and utilization of the control (unheated) lactalbumin is complete. The calculated digestibility and utilization are based, therefore, on unheated protein as representing a value of 100%. By multiplying the biological value by the digestibility, a quantitative expression is obtained which Cahill, Schroeder and Smith ('44) have termed the "nutritive index." This value expresses more accurately the over-all nutritive value of a protein than does either the digestibility or biological value alone. For each heated protein fed, the averages of the data of the two experimental periods have been used for purposes of calculation. The averages of the data from the three control periods which delimit and separate the experimental periods have been similarly used.

In table 2 are listed the average per cent nitrogen output in the feces and the calculated digestibilities of all the heated proteins. It is apparent that the extent of absorption of the nitrogen of the heated proteins depends upon the method of heating and upon the length of the heating period. Dry-heating of commercial lactalbumin lowers the availability of the protein nitrogen only slightly, whereas if it is autoclaved the digestibility is markedly diminished. This decrease is directly proportional to the length of the heating period. Of special significance is the fact that the digestibility of autoclaved extracted lactalbumin was considerably higher than that of the autoclaved unextracted product. The absorption of the 30-min. autoclaved extracted protein was almost as complete as that of the unheated materials.

The average biological values and nutritive indices are shown in table 2. It will be noted that in all cases the utiliza-

tion of the absorbed nitrogen from the heated protein was as good as or better than that from the unheated protein. It is evident that no loss in nutritional quality was caused by the dry-heating process or by autoclaving extracted lactalbumin for 30 min. On the other hand, if commercial lactalbumin is autoclaved for 30 min. the nutritive index, which

TABLE 2

Digestibility, fecal nitrogen, biological value and sugar content of heated lactalbumin as related to nutritive index

	DIGESTIBILITY [1]	NITROGEN OUTPUT IN FECES		BIOLOGICAL VALUE [2]	NUTRITIVE INDEX [3]	SUGAR CONTENT
		Control	Exptl.			
	%	%	%	%	%	%
Commercial lactalbumin, dry-heated 120°C., 60 min.	95	17	23	109	103	4.0
Commercial lactalbumin, autoclaved 30 min.	84	18	31	107	90	1.2
Extracted lactalbumin, autoclaved 30 min.	97	17	20	104	100	0.3
Commercial lactalbumin, autoclaved 60 min.	72	16	36	111	80	0.8
Extracted lactalbumin, autoclaved 60 min.	80	17	33	107	85	0.3
Extracted lactalbumin + 5% lactose, autoclaved 60 min.	63	19	45	115	73	0.9

[1] Digestibility (per cent) $= \frac{\text{Food N absorbed}}{\text{Total food N}} \times 100.$

$$\text{Food N absorbed} = \text{Total food N} - \left(\begin{matrix}\text{Experimental}\\ \text{fecal N}\end{matrix} - \begin{matrix}\text{Control}\\ \text{fecal N}\end{matrix}\right).$$

[2] Biological value (per cent) $= \dfrac{\text{Food N absorbed} - \left(\begin{matrix}\text{Experimental}\\ \text{urinary N}\end{matrix} - \begin{matrix}\text{Control}\\ \text{urinary N}\end{matrix}\right)}{\text{Food N absorbed}} \times 100.$

[3] Nutritive index = biological value × digestibility.

involves the factor of digestibility, is lowered by 10%. When the autoclaving period is extended to 60 min. the damage incurred by both commercial and extracted lactalbumin is considerable, though again the extracted protein is not so severely injured as is the commercial product with its higher content of carbohydrate.

It was shown in the balance study that the removal of reducing substances from commercial lactalbumin prior to the heating process markedly affects the nutritive value of the protein. It was therefore desirable to determine quantitatively the reducing sugar content of all the proteins used, in an attempt to correlate these results with the *in vivo* studies. A modification of the Shaffer and Hartmann copper reduction iodometric method for the determination of lactose in milk was used (Winton and Winton, '45). The results of these analyses (table 2) show that the recovery of lactose from commercial lactalbumin is markedly reduced after it has been autoclaved. The sugar content of the protein is decreased approximately 75 to 85% by autoclaving the material for 30 and 60 min., whereas the dry-heating process removes only 20% of the sugar. The marked loss of reducing sugar content is not due to the heat treatment per se, for if lactose is autoclaved with a finely divided inert material, such as talc, about 85% of the sugar is still found to be present after autoclaving. This suggests that a reaction between the carbohydrate and the protein has occurred.

DISCUSSION

The ability of lactalbumin to maintain nitrogen balance in the adult dog is impaired when the protein has been heated. This is in accord with the findings of many investigators who have used other proteins and different species of experimental animals. It has been reported that there is greater damage to the nutritive value of a protein as the length of the heating period is increased (Morgan and Kern, '34; Seegers and Mattill, '35; Davis, Rizzo and Smith, '49). This has been found to be true in the present study. It appears that the detrimental effect of heat is gradual and progressive.

Moisture and temperature increases accelerate the changes which occur in the protein as it is heated. In the present study, dry-heating of commercial lactalbumin produced very little alteration in the protein, judging from the nutritive in-

dex, whereas autoclaved protein was markedly inferior even when the duration of the heat treatment was less.

Recent work has shown that the reaction between reducing sugars and protein may be responsible for the decreased nutritive value of heated proteins (Patton et al., '48a, '48b; Hill and Patton, '47; Stevens and McGinnis, '47). The results of the present investigation support this hypothesis. Heated extracted lactalbumin differs markedly from heated commercial lactalbumin in its ability to maintain nitrogen balance in the adult dog. Whereas autoclaving the commercial product for 30 min. produces a material which is unable to support nitrogen equilibrium, the same treatment of extracted lactalbumin causes no apparent change as regards nitrogen availability. If the duration of heat treatment is increased to 60 min. the extracted protein becomes less adequate but its nutritive value is nevertheless greater than that of commercial lactalbumin subjected to the same treatment.

A critical analysis of the data shows that a change in the digestibility of the heated proteins can account for a major portion of the damage incurred. Morgan and co-workers ('26, '34) reported that the heating process does not affect the digestibility of a protein, though its biological value may be impaired. More recently Weast, Groody and Morgan ('48) observed that heated casein is not well absorbed by the growing dog. This is in direct contrast to the report by Morgan ('31) of large urinary nitrogen losses in rats fed heated protein. Others have found, however, that in this species the digestibility of heated protein is not as complete as that of unheated protein (Chick et al., '35; Davis, Rizzo and Smith, '49).

Paradoxically, the real biological value of all proteins used in the present experiments was uniformly high, indicating that whatever nitrogen the dogs were able to absorb was well utilized. Apparently the animals neither absorbed atypical peptides which were excreted in the urine, nor wasted absorbed nitrogen because some essential amino acid was

lacking, thereby preventing mutual simultaneous supplementation.

Our observations with respect to nitrogen balances and the carbohydrate content of the various proteins support the hypothesis that the browning reaction which occurs when protein is heated is some type of condensation between nitrogenous compounds and reducing sugars, specifically involving the amino groups of the protein and the free aldehyde groups of the carbohydrate. While the presence of carbohydrate in commercial lactalbumin has been shown to play a prominent role with regard to the nutritive value of the protein when it is subjected to heat treatment, it should be emphasized that this phenomenon is probably but one of many complex and little understood reactions which occur. Actual destruction of amino acids when casein or soybean oil meal is heated in the presence of carbohydrate has been reported (Patton et al., '48a, '48b). The heat treatment itself undoubtedly causes changes in the protein molecule, especially at high temperatures (Mecham and Olcott, '47). Decomposition of proteins becomes extensive at temperatures of 150°C. or more, with a loss of considerable amounts of ammonia, decreases in amino and basic groups, and changes in solubility.

It would appear, nevertheless, that an interaction of a protein molecule and a reducing sugar can account for much of the damage which occurs when commercial lactalbumin is heated. This reaction is particularly noticeable at relatively low temperatures and with comparatively short periods of heating. As the duration of heating is prolonged or the temperature increased, other reactions obscure this phenomenon.

SUMMARY

No change in its nutritional quality occurred when commercial lactalbumin was dry-heated at 120°C. for 60 min. Its digestibility and nutritive index were found to be significantly decreased by autoclaving. This decrease in nutritive value was in proportion to the severity of the heat

treatment. The nutritional quality of extracted lactalbumin was not as seriously impaired by comparable heat treatment. The latter was reconstituted with lactose with respect to the carbohydrate content of the original protein. Upon heating, the reducing sugar content diminished and severe negative nitrogen balances resulted. Decreased digestibility, as evidenced by increased fecal nitrogen, accounted for the decrease in nutritive value. Utilization of absorbed nitrogen remained uniformly high in all cases.

LITERATURE CITED

BORSOOK, H., AND H. WASTENEYS 1925 The interaction of free amino-nitrogen and glucose. Biochem. J., *19:* 1128.

CAHILL, W. M., L. J. SCHROEDER AND A. H. SMITH 1944 Digestibility and biological value of soybean protein in whole soybeans, soybean flour and soybean milk. J. Nutrition, *28:* 209.

CHICK, H., M. A. BOAS-FIXSEN, J. C. D. HUTCHINSON AND H. M. JACKSON 1935 The influence of variation in the level of protein in the diet and of heating the protein on its biological value. Biochem. J., *29:* 1712.

DAVIS, R. M., P. RIZZO AND A. H. SMITH 1949 The effect of heat on the nutritive value of lactalbumin. I. Growth on diets containing heated proteins. J. Nutrition, *37:* 115.

DEVLIN, H. B., AND C. A. ZITTLE 1944 A nutritional study of human globin in rats. J. Biol. Chem., *156:* 393.

EVANS, R. J., J. MCGINNIS AND J. L. ST. JOHN 1947 The influence of autoclaving soybean oil meal on the digestibility of the proteins. J. Nutrition, *33:* 661.

FAIRBANKS, B. W., AND H. H. MITCHELL 1935 The nutritive value of skim-milk powders, with special reference to the sensitivity of milk proteins to heat. J. Agr. Res., *51:* 1107.

HILL, E. G., AND A. A. PATTON 1947 The Maillard reaction in microbiological assay. Science, *105:* 481.

KUETHER, C. A., AND V. C. MYERS 1948 The nutritive value of cereal proteins in human subjects. J. Nutrition, *35:* 651.

MAILLARD, L. C. 1912 Action des acides aminés sur les sucres; formation des melanoidines par voie methodique. Compt. rend. Acad. d. sci., *154:* 66.

———— 1912 Reaction generale des acides aminés sur les sucres: ses conséquences biologique. Compt. rend. soc. biol., *72:* 599.

MAYNARD, L. A., R. C. BENDER AND C. M. MCCAY 1932 Vitamin A and protein content of various fish meals. J. Agr. Res., *44:* 591.

MCCOLLUM, E. V., AND M. DAVIS 1915 The cause of the loss of nutritive efficiency of heated milk. J. Biol. Chem., *23:* 247.

MECHAM, D. K., AND H. S. OLCOTT 1947 Effect of dry heat on proteins. Ind. Eng. Chem., *39:* 1023.

MITCHELL, H. H., W. BURROUGHS AND J. R. BEADLES 1936 The significance and accuracy of biological values of proteins computed from nitrogen metabolism data. J. Nutrition, *11:* 257.

MORGAN, A. F. 1931 The effect of heat upon the biological value of cereal proteins and casein. J. Biol. Chem., *90:* 771.

MORGAN, A. F., AND G. E. KERN 1934 The effect of heat upon the biological value of meat proteins. J. Nutrition, *7:* 367.

MORGAN, A. F., AND B. F. KING 1926 Changes in biological value of cereal proteins due to heat treatment. Proc. Soc. Exp. Biol. Med., *23:* 353.

MORGAN, A. F., AND V. A. PETRO 1930 The effect of heat treatment upon the biological value of proteins. J. Home Econ., *22:* 857.

MUMFORD, H. W. 1933–34 Nutritive Values of Dried Skim Milk are Studied. Illinois Agr. Exp. Sta., 47th Annual Report, p. 82.

MURLIN, J. R., M. E. MARSHALL AND C. D. KOCHAKIAN 1941 Digestibility and biological value of whole wheat breads as compared with white bread. J. Nutrition, *22:* 573.

MURLIN, J. R., E. S. NASSET AND M. E. MARSH 1938 The egg-replacement value of the proteins of cereal breakfast foods, with a consideration of heat injury. Ibid., *16:* 249.

OLCOTT, H. S., AND T. D. FONTAINE 1941 The effect of autoclaving on the nutritive value of the proteins in cottonseed meal. Ibid., *22:* 431.

ORTEN, J. M., AND A. H. SMITH 1937 A study of certain metabolites and related compounds as precursors of endogenous citric acid. J. Biol. Chem., *117:* 555.

PATTON, A. R., AND E. G. HILL 1948 Inactivation of nutrients by heating with glucose. Science, *107:* 68.

PATTON, A. R., E. G. HILL AND E. M. FOREMAN 1948a Amino acid impairment in casein heated with glucose. Ibid., *107:* 623.

———— 1948b The effect of browning on the essential amino acid content of soy globulin. Ibid., *108:* 659.

PATTON, A. R., AND W. E. PYKE 1946 The role of amino acids and glucose in the browning of potato chips and dehydrated potatoes. Abstracts, A.C.S. Meeting, Chicago, Division of Agricultural and Food Chemistry, Sept.

RANSEY, R. J., P. H. TRACY AND H. A. RUEHE 1933 The use of corn sugar in the manufacture of sweetened condensed skim-milk. J. Dairy Sci., *16:* 17.

RIESEN, W. H., D. R. CLANDININ, C. A. ELVEHJEM AND W. W. CRAVENS 1947 Liberation of essential amino acids from raw, properly heated, and overheated soy bean oil meal. J. Biol. Chem., *167:* 143.

ROSS, A. F. 1948 Deterioration of processed potatoes. Advances in Food Research, Vol. I., Ed. by E. M. Mrak and G. F. Stewart. Academic Press, Inc., New York, New York, p. 257.

SCHNEIDER, B. H. 1932 Nitrogen-balance studies with various fish meals. J. Agr. Res., *44:* 723.

SEEGERS, W. H., AND H. A. MATTILL 1935 The nutritive value of animal tissues in growth, reproduction and lactation. J. Nutrition, *10:* 271.

STADTMAN, E. R. 1948 Non-enzymatic browning in fruit products. Advances in Food Research, Vol. I, Ed. by E. M. Mrak and G. F. Stewart. Academic Press, Inc., New York, New York, p. 325.

STEVENS, J. M., AND J. MCGINNIS 1947 The effect of autoclaving lysine in the presence of carbohydrate on its utilization by the chick. J. Biol. Chem., *171:* 431.

WAISMAN, H. A., AND C. A. ELVEHJEM 1938 The effect of autoclaving on the nutritive value of edestin. J. Nutrition, *16:* 103.

WEAST, E. O., M. GROODY AND A. F. MORGAN 1948 Utilization by dogs of the nitrogen of heated casein. Am. J. Physiol., *152:* 286.

WINTON, A. L., AND K. B. WINTON 1945 The Analysis of Foods. John Wiley and Sons, Inc., New York, New York, p. 746.

WOODS, E., W. M. BEESON AND D. W. BOLIN 1943 Field peas as a source of protein for growth. J. Nutrition, *26:* 327.

EFFECTS OF DIETARY DEPLETION OF RIBOFLAVIN [1]

M. K. HORWITT, O. W. HILLS,[2] C. C. HARVEY, E. LIEBERT AND D. L. STEINBERG

Biochemical Research Laboratory, Elgin State Hospital, Elgin, Illinois

ONE FIGURE

(Received for publication July 12, 1949)

Stannus ('13) described many of the clinical symptoms of ariboflavinosis more than 20 years before riboflavin was discovered. Subsequent reports produced additional evidence of the dietary nature of these symptoms (Bahr, '14; Scott, '18). The specificity of some of the clinical signs received scientific confirmation during the period when riboflavin was being concentrated and isolated (Goldberger and Tanner, '25; Moore, '30; Landor and Pallister, '35). The availability of riboflavin made possible the human dietary experiments of Sebrell and Butler ('38), in which lesions attributable to riboflavin deficiency were produced by a regimen containing 0.5 mg of riboflavin per day. Since then much work has been done by many able investigators, but there remains considerable controversy as to what is, or is not, riboflavin deficiency.

Restricting this discussion to attempts to produce signs of pure riboflavin deficiency by means of dietary experiments of

[1] Supported by research grants from the National Vitamin Foundation and the U. S. Public Health Service. Sponsored by the National Research Council, Food and Nutrition Board, Committee on Vitamin Deficiency Studies, at Elgin State Hospital; Dr. R. M. Wilder, Chairman, Dr. E. S. G. Barron, Dr. C. A. Elvehjem, Dr. M. K. Horwitt, Dr. A. C. Ivy, Dr. Erich Liebert, Dr. D. L. Steinberg, Dr. Derrick Vail, Dr. Ray D. Williams.

[2] U. S. Public Health Service Senior Fellow, 1947.

at least 5 months' duration, it should be noted that recent efforts to produce such an uncomplicated syndrome have not yielded positive results. Williams, Mason, Cusick and Wilder ('43) fed a diet which provided between 0.8 and 0.9 mg (0.35 mg per 1,000 cal.) of riboflavin per day for over 9 months, but no clinical evidence of riboflavin deficiency was produced in that time. Keys, Henschel, Mickelsen, Brozek and Crawford ('44) observed no signs of deficiency on a diet containing 1.0 mg (0.3 mg per 1,000 cal.) of riboflavin per day, fed for 5 months. Horwitt, Liebert, Kreisler, and Wittman ('48) fed a diet which contained between 0.8 and 0.9 mg of riboflavin per day for over two years, and only one subject out of the 22 observed during this period showed any signs that might be attributed to lack of riboflavin. Although in all of these studies the urinary excretion of riboflavin was reduced to minimum levels, the dermatological signs reported by Sebrell and Butler ('38) were not positively reproduced.

Can a pure riboflavin deficiency be produced experimentally? If skin lesions are observed during subsistence on diets low in riboflavin, are these lesions specific signs of riboflavin deficiency? It was decided to try to answer these questions by feeding for over a year a diet containing between 0.5 and 0.6 mg of riboflavin per day. The choice of this level was influenced by the positive results reported by Sebrell and Butler ('38) on 0.5 mg per day and the negative results of more recent workers who fed larger amounts.

METHODS OF STUDY

The techniques employed were strongly influenced by the experiences reported by Williams, Mason, Cusick and Wilder ('43). *The selection of subjects* was made in a manner quite similar to that reported by Horwitt, Liebert, Kreisler and Wittman ('48). The subjects for the study were chosen after evaluating the qualities of several hundred mental patients. Choice was based upon chronicity of mental illness, excellence of physical condition, and the presence of a reasonable amount of emotional stability. The psychiatric diagnoses

varied widely, which factor minimized the very unlikely possibility that a metabolic disorder concerned with riboflavin could be related to the mental disorder of the subjects. Choice of subjects was also influenced by attitudes, cooperativeness, physical health and habits of eating. After a three-month trial feeding period on a diet which was made up to resemble that which was to be used later but which provided about twice as much riboflavin (1.1 mg per day), 39 subjects were selected. They were divided into three groups in May, 1947. Group A, 15 subjects, received a diet supplying 0.55 mg of riboflavin per day; group B, 14 subjects, received the same diet but in addition were given a tablet containing 2 mg of riboflavin per day; group C, 10 subjects, ate the regular hospital diet ad libitum. Only one subject (B6) failed to cooperate in eating and he was in the supplemented group. Another subject (B2) was not available for further observation after 8 months of supplementation.

The tentative original plan called for experimental periods of at least one year's duration before riboflavin would be added to the diet of the depleted group. However, the appearance in three subjects of symptoms of dermal lesions necessitated supplementing the diet of these three during the 9th and 10th months of the period of depletion.

Diet and dietary supplements

The details of the diets and the extensive dietary controls are described elsewhere (Horwitt, Sampson, Hills and Steinberg, '49). In brief, three separate lists of foods, which permitted the serving of menus that were repeated every 9 days, were fed by a kitchen staff of 4 full-time workers in a building especially remodeled for nutritional investigations. The basic diet provided 2,200 cal., 57 gm protein, 65 gm fat and 0.55 mg of riboflavin per day. The natural foods in the diet provided less than 45 gm of protein per day, and it was necessary to add from 10 to 15 gm of calcium caseinate [3] to the

[3] Supplied by the Borden Company.

daily menus in order to avoid the theoretical possibility of an amino acid deficiency.

With one day's standard food ration regarded as 100%, the amount of food was varied with the subject's size and appetite, so that 6 subjects in group A received more or less than 100%. These were subjects A1, A4, A5, A7, A9 and A15, who received 110, 110, 80, 110, 120 and 120%, respectively. All foods were repeated every three days, but the form in which the food was served was varied in a 9-day cycle.

The diet containing approximately 1.1 mg of riboflavin per day which was fed during the three-month preliminary period was similar to the one used in the later experimental period except that the allotment of meat was slightly larger and a flour of higher riboflavin content was used to make the bread.

The following daily supplement of vitamin [4] was given to all subjects on the controlled diets: thiamine hydrochloride 0.6 mg, niacinamide 10 mg, pyridoxine 1 mg, calcium pantothenate 3 mg, biotin 0.05 mg, folic acid 0.1 mg, ascorbic acid 50 mg, vitamin A 4,000 U.S.P. units, and vitamin D 400 U.S.P. units. In addition, 500 mg of dicalcium phosphate were given daily and 180 mg of ferrous sulphate three times weekly. Calculation of the intake of riboflavin on the 0.55 mg diet of the A group on the basis of body weight gives a figure of approximately 9.0 μg per kilogram of body weight per day. Analytical control of the riboflavin content of the diet was continuous during the first year of the study. About one-half of all the meals were sampled and assayed by both fluorimetric (Slater and Morell, '46) and microbiological methods (Roberts and Snell, '46).

Tests employed

Since an earlier study (Horwitt and Kreisler, '49) using a diet containing 0.8 to 0.9 mg of riboflavin and 0.4 mg of thiamine per day had produced definite changes in carbohy-

[4] Special formula supplied by Hoffmann-LaRoche, Inc.

drate metabolism, it was considered important to repeat some of this work in the present study, in which the allowance of riboflavin was decreased to 0.55 and that of thiamine raised to approximately 0.9 mg per day. The levels in the blood of *glucose, lactic acid and pyruvic acid* were determined before and after oral glucose administration and after a mild exercise test, which was given 60 min. after the ingestion of the glucose. This test, the methods used in the analyses and the use of the results to obtain an index of carbohydrate metabolism have been described by Horwitt and Kreisler ('49).

The 24-hour *urinary excretion* of riboflavin and the excretion of riboflavin during the 4 hours following the injection of 1 mg of riboflavin are described by Horwitt et al. ('49). Blood riboflavin was estimated according to a microbiological method similar to that of Strong, Feeney, Moore and Parsons ('41).

Hematological analyses included determinations of hemoglobin, hematocrit, red blood cell and differential counts. Basal metabolic rates, electrocardiograms, electroencephalograms and orthodiagrams of heart size were obtained before, during and after the periods of dietary depletion. Cephalin-cholesterol flocculation tests, icteric indices and the blood levels of cholesterol, N.P.N., creatinine and phospholipids were also determined.

The large number of *clinical examinations* which were conducted daily, or as often as was necessary, will be described in detail in a paper dealing with this aspect of the experiments. They included neurological tests of reflexes, vibration sense and auditory acuity; tests of visual acuity, flicker fusion, tear flow, dark adaptation, corneal sensitivity and internal ocular pressure; and slit-lamp examination for corneal vascularization. A photographic record was kept of the gait, face, tongue, lips, genitals and finger capillaries. The ability of the subjects to perceive flicker was determined by using a mercury bulb which was illuminated and extinguished at different controlled rates. The point at which the light interruptions were just recognized by the subject was accepted

as the threshold of flicker fusion. Pupil size and pupil contraction time were also determined photographically. Psychiatric evaluations and the psychological testing of behavior were a part of the routine; these will be reported elsewhere.

Growth and wound healing of rats

As an additional test of the riboflavin content of the basal diet, homogenized aliquots (with additional iron provided by adding 0.17 gm ferric ammonium sulphate to 100% of the diet) were fed to rats. In a typical experiment 22 weanling rats were divided into two groups. Half of these animals received the basal diet fed to the human subjects in group A and the other half the same diet with 60 μg of riboflavin added per day per rat. Simultaneously, in order to facilitate the availability of animals with marked deficiencies of riboflavin, a diet containing half as much riboflavin as that provided for the subjects in group A was fed to another group of 18 rats. The latter diet differed from the A group diet in that all meat and bread were removed and an equivalent amount of protein in the form of lactalbumin [5] added. This diet provided approximately 300 μg of riboflavin for each 100% of the diet, or 0.5 μg per gram of dry weight. Half of the animals on this diet received 60 μg of riboflavin daily as a supplement.

While attempting to evaluate the effect of riboflavin on the dermal lesions observed in humans, preliminary studies of wound healing were made on 6 150-day-old rats, 4 of which were on the deficient diet and two of which received supplementary riboflavin daily. Circular wounds 1 cm in diameter were made on the dorsal surfaces of the rats by removing the skin to the depth of its normal separation from underlying tissue, according to the technique described by Paul, Paul, Taylor and Marsters ('45). The rate and character of wound healing were observed and compared with the amount of riboflavin in the diets of the animals.

[5] Supplied by the Borden Company.

OBSERVATIONS

Blood glucose, lactic acid and pyruvic acid

These data for the subjects in groups A and B are given in table 1. The indices of carbohydrate metabolism (CMI) were calculated on the basis of the analysis of 66-minute samples, obtained 5 minutes after 1 minute of stair climbing, done 60 minutes after the oral administration of glucose. Unlike the results of the previously reported study of thiamine deficiency (Horwitt and Kreisler, '49), there were no apparent changes in the carbohydrate metabolism; i.e., no accumulation of lactic and pyruvic acids in the blood. The data of the completed group compare favorably with those previously published and obtained with subjects who had received diets which were adequate in all respects.

Urinary excretion of riboflavin

These details are reported elsewhere by Horwitt et al. ('49). In brief, the average subject dropped from an original excretion of 400 μg in 24 hours, on the hospital diet which contained 1.6 mg per day, to an average of 112 μg on the 1.1 mg diet used in the preliminary period. Seven days after the 0.55 mg diet was started, the average excretion of the subjects of group A dropped to 50 μg; it persisted at that level for 9 months and then fell to as little as 36 μg per day. Excretions after supplementation with riboflavin depended upon the size of the supplement. Thus, when 2 mg were given once, twice or three times a day, tissue saturation as determined by the 4-hour excretion test was obtained at 38, 25 and 11 days, respectively.

The many analyses of the constituents of the blood did not reveal any differences that could be interpreted as being related to deficiency of riboflavin. The hematological examinations and liver function tests all gave results which are considered to be within "normal" ranges. No evidence of pathological findings was noted in examination of the electrocardiograms, heart orthodiagrams or basal metabolic rates.

TABLE 1

Average glucose, lactate and pyruvate after glucose administration and exercise (milligram per 100 ml of blood)

TEST		TIME OF TEST					
		April '47	July '47	Sept. '47	Nov. '47	Mar. '48	Aug. '48
				Group A			
Basal	Glucose	102 ± 7	102 ± 10	103 ± 10	106 ± 7	98 ± 7	112 ± 9
	Lactate	8.0 ± 2.1	9.4 ± 2.5	8.6 ± 2.1	7.7 ± 1.6	7.5 ± 1.8	7.9 ± 2.4
	Pyruvate	0.93 ± 0.18	0.94 ± 0.24	0.85 ± 0.22	0.85 ± 0.14	0.88 ± 0.16	0.85 ± 0.20
60 minutes	Glucose	155 ± 23	142 ± 16	157 ± 30	163 ± 32	144 ± 28	159 ± 31
	Lactate	13.5 ± 3.2	12.5 ± 2.3	14.2 ± 2.2	13.9 ± 2.8	12.1 ± 4.1	14.3 ± 4.0
	Pyruvate	1.38 ± 0.22	1.24 ± 0.24	1.43 ± 0.26	1.32 ± 0.28	1.29 ± 0.13	1.44 ± 0.20
66 minutes [1]	Glucose	140 ± 18	133 ± 22	145 ± 28	144 ± 26	148 ± 51	149 ± 34
	Lactate	18.7 ± 2.8	18.8 ± 3.2	21.0 ± 5.0	18.8 ± 3.3	18.6 ± 6.5	20.6 ± 4.2
	Pyruvate	1.63 ± 0.23	1.57 ± 0.26	1.56 ± 0.26	1.45 ± 0.22	1.51 ± 0.22	1.75 ± 0.34
CMI [2]		7.6 ± 3.1	8.0 ± 4.6	7.7 ± 5.1	6.1 ± 3.5	7.0 ± 4.0	8.4 ± 5.5
120 minutes	Glucose	121 ± 23	120 ± 23	132 ± 22	131 ± 18	123 ± 15	140 ± 31
	Lactate	14.3 ± 3.4	14.4 ± 3.8	13.2 ±2.9	13.1 ± 1.5	13.3 ± 2.5	13.6 ± 2.5
	Pyruvate	1.23 ± 0.17	1.24 ± 0.25	1.23 ± 0.28	1.16 ± 0.27	1.15 ± 0.22	1.22 ± 0.25
				Group B			
Basal	Glucose	102 ± 8	96 ± 5	104 ± 9	106 ± 4	98 ± 4	107 ± 5
	Lactate	7.1 ± 1.6	9.1 ± 2.2	8.3 ± 1.6	7.3 ± 1.7	8.2 ± 1.6	7.8 ± 2.0
	Pyruvate	0.91 ± 0.22	0.92 ± 0.21	0.77 ± 0.17	0.97 ± 0.20	0.86 ± 0.16	0.93 ± 0.18
60 minutes	Glucose	142 ± 30	141 ± 28	144 ± 22	156 ± 25	155 ± 23	145 ± 27
	Lactate	13.5 ± 3.0	13.6 ± 3.0	14.6 ± 3.0	13.0 ± 2.7	13.7 ± 4.3	14.0 ± 2.6
	Pyruvate	1.33 ± 0.25	1.33 ± 0.28	1.22 ± 0.28	1.32 ± 0.26	1.35 ± 0.21	1.47 ± 0.21
66 minutes [1]	Glucose	137 ± 26	134 ± 23	135 ± 21	144 ± 28	140 ± 20	131 ± 22
	Lactate	19.3 ± 4.2	18.3 ± 4.1	19.6 ± 4.4	18.3 ± 2.4	20.5 ± 4.0	18.9 ± 4.2
	Pyruvate	1.66 ± 0.28	1.48 ± 0.29	1.50 ± 0.25	1.66 ± 0.22	1.67 ± 0.41	1.85 ± 0.44
CMI [2]		7.9 ± 2.4	6.8 ± 3.3	7.6 ± 2.8	7.2 ± 2.9	8.8 ± 3.5	9.8 ± 2.9
120 minutes	Glucose	131 ± 29	119 ± 26	130 ± 22	126 ± 17	124 ± 19	121 ± 19
	Lactate	12.8 ± 2.8	12.6 ± 3.5	11.2 ± 1.9	12.8 ± 2.7	11.5 ± 1.9	12.2 ± 3.5
	Pyruvate	1.24 ± 0.18	1.18 ± 0.37	1.12 ± 0.28	1.12 ± 0.20	1.09 ± 0.21	1.13 ± 0.27

[1] Immediately after the 60-minute blood sample is drawn, the subject walks up and down a flight of stairs for one minute. Five minutes later a blood sample is drawn, and the latter is referred to as the 66-minute sample.

[2] The glucose, lactic and pyruvic acid data observed from the 66-minute sample are calculated according to the formula: $CMI = \frac{L - \frac{G}{10} + 15\,P - \frac{G}{10}}{2}$, where G, L and P are glucose, lactic acid and pyruvic acids in milligrams per cent, respectively.

Toward the end of the deficiency period psychological tests of mental fatigue produced results which indicated that the subjects in group A were less efficient than the subjects in the supplemented group. These tests will be repeated after group A has been supplemented with riboflavin for one year, at which time they will be evaluated.[6]

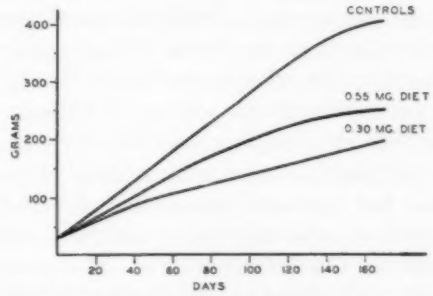


Fig. 1 The growth of rats on the human experimental diets. The 0.55 mg diet was the homogenized equivalent of the food fed to group A subjects; the 0.30 mg diet was similar to the latter except that meat and bread were removed and an equivalent amount of protein added as lactalbumin. The controls received the same diets but 60 μg of riboflavin were added daily. The controls for the two diets are plotted together because the results obtained when riboflavin was added were very similar.

Experiments with rats

That the group A diet was inadequate with respect to riboflavin for the optimum growth of rats is shown in figure 1. Good growth was obtained when riboflavin was added to the diet.

Of possible significance is the observation, common to most nutritional deficiency studies, that though the deficient animals had more skin lesions around the head, such lesions were not completely absent in the control group. However, in the latter group any such lesion would heal soon after appearing, whereas a similar one would tend to persist in the animal on the deficient diet.

[6] These psychological observations will be reported by Miss Thelma Johnson.

The healing of the circular wounds produced in the rats was directly related to the amount of riboflavin in their diets. There was a marked difference in the rates of tissue proliferation in the rats fed different levels of riboflavin. The wounds of the animals who received added riboflavin developed a scab the first day and decreased in size in three days. At 14 days only a tiny crust was left, which was lost at 20 days. No sign of previous injury was detectable at 28 days, at which time there was complete regrowth of hair. The wounds of animals on the A group diet healed more slowly. There was some wound contraction on the 4th day but the area was quite moist. On the 28th day healing was far from complete, and after 54 days the wounds were still evident, with marked retardation of the fur regrowth. Bosse and Axelrod ('48) made similar observations with respect to rat diets much more deficient in riboflavin than those we fed. The present preliminary rat study with diets of borderline deficiency will be repeated, using larger numbers of animals and paired-feeding techniques.

Clinical examinations

Cheilosis,[7] involving the vermillion border of the lips and including vertical fissuring, crusting and desquamation, was seen in only one subject (A15). In his case the lesion was never more than minimum, and was manifested only by redness and slight vertical fissuring in a lip that was naturally almost abnormally protruberant. Angular stomatitis, the crusting and fissuring of the oral angles, appeared for varying periods of time in three members (A1, A5 and A11) of the depleted group but in none of the supplemented subjects. In none of these three was there evidence of excessive salivation, or of inefficient dentures. In the case of subject A1, a bacteriological investigation of the wound produced no evidence of a mycotic infection.

The first manifestation of possible deficiency of riboflavin appeared in subject A1 in September 1947, 4 months after

[7] Dr. Cleveland J. White and Dr. H. S. Steinberg gave dermatological advice and treatment during the period of observation of the skin lesions.

the beginning of the 0.55 mg diet. This consisted of a lesion, which was diagnosed as typical herpes simplex vesicle, at the left oral angle. During the succeeding 6 weeks a definite horizontal fissure with crusting appeared at both oral angles. Local therapy with zinc oxide and camphorated oil assisted healing of the right oral angle in less than three weeks. That on the left, however, remained in a fluctuating state of remission and exacerbation and did not heal completely until after riboflavin was administered. In addition to the angular stomatitis, subject A1 had a scaling, crusting lesion of the scalp which was resistant to routine therapy with sal-sulphur ointment. A similar condition was present in the hairy areas of the chest. On the face, severe vertical fissuring appeared at the naso-labial folds and eroded through the muco-cutaneous border into the nostrils. Small fissures also appeared at the orifice of the nares. All of these conditions healed very rapidly after the administration of riboflavin.

The second subject (A5) developed similar lesions without the appearance of any pre-existing herpes. They were noted in March 1948, two weeks after the appearance of a severe scrotal dermititis (see below). Because of the severity of the latter lesion, the patient was given 6 mg of riboflavin per day. This supplementation was begun 5 days after the appearance of small fissures at both oral angles. Marked improvement was observed within 48 hours and the lesions healed completely in two weeks. It may be significant that this subject was the only poor eater in group A. He maintained his weight of 67 kg on an 80% diet, and so received only 0.44 mg of riboflavin per day.

In the third subject (A11) to develop an angular fissure, it healed without therapy. The lesion had been present constantly in varying degrees for 5 weeks. There was no recurrence.

Scrotal skin changes

The appearance of redness, scaling and desquamations of the superficial layer of the skin of the scrotum was first

observed in subject A8 in the 8th month of the 0.55 mg diet. The skin of the anterior surface of the scrotum was markedly reddened. The lesion was discrete, bilateral, and not connected in mid-line. A similar lesion appeared in 12 of the 15 subjects in group A between the 8th and 10th month after the beginning of the 0.55 mg diet. It was most severe in subject A5, appearing markedly reddened, raw and weeping, and extending to the adjacent areas of the thighs. Elevation of the scrotum and warm saline applications ameliorated the acute symptoms but marked erythema persisted, followed by induration of the skin. Three weeks after the onset of this condition the administration of riboflavin produced striking improvement within 48 hours, and all signs of inflammation, except for residual erythema, disappeared within a week. The marked scrotal lesion of subject A8 responded equally dramatically within one week after the administration of riboflavin. This lesion had been present for 6 weeks with little change before riboflavin was administered.

The remaining 10 subjects showed various degrees of the lesions described, i.e., moderately severe to mild scrotal erythema and scaling. Only a few of them complained of itching.

After all of the lesions had appeared in the A group, three subjects in the B group developed a scrotal erythema with little or no scaling. This prompted an investigation of several hundred other hospital patients, who presumably were adequately nourished. Scrotal erythema, but no scaling, was found in a large percentage of those who were incontinent. The basic irritation of the skin of the subjects deficient in riboflavin was probably explained by poor hygiene. However, no bacterial or mycotic infection could be found. In the absence of adequate riboflavin, tissue repair became retarded and thereby the development of the more severe manifestations of scrotal dermatitis was promoted. The possibility that cleaning agents used in the hospital laundry may have been a contributing factor was considered but not established.

Flicker fusion

The threshold for flicker fusion, when tested at a distance of 6 feet from the source of flicker, showed an increase in three subjects in group A: A4, A6 and A12. None of the subjects in group B showed similar changes. When in September 1948, the testing was done at a distance of 12 feet from the source of flicker, all but one of the A group showed a marked increase as compared to the responses of the subjects in group B.

DISCUSSION

The report of Sebrell and Butler ('38) that signs of deficiency of riboflavin can be obtained at a riboflavin level of 0.5 mg per day has been confirmed by the above observations, in which angular stomatitis was obtained at a level of 0.6 mg or less per day. The scrotal dermatitis we observed may be similar to that reported on the severe vitamin B complex deficiency diet used by Berryman et al. ('47). Whether or not these and the other abnormalities observed are specific signs of riboflavin deficiency is not certain. That riboflavin is necessary for the growth of tissue is generally agreed. Any local trauma to the skin must be repaired by new growth. Will not the signs of deficiency, then, be partially dependent upon the need for riboflavin for local tissue replacement? If this is so, then the location of disorders of the skin will depend upon the presence of local irritations, infections and other traumas as primary causes. Thus a slight sore, originally diagnosed as a common "herpes simplex," became a typical angular stomatitis; an itchy scalp became a seborrheic dermatitis; and a sore nose which appeared in conjunction with a mild upper respiratory infection became inflamed, the inflammation spreading over the anterior portion of the septum and a fissure appearing at the naso-labial fold. All these lesions were improved spectacularly when riboflavin was administered after other therapy had failed. The same can be said of the scrotal dermatitis which was so prevalent in the subjects of group A, although the primary cause of this

particular lesion remains unknown. It may be that many of the "classical" signs of riboflavin deficiency are subject to controversy because the different patients observed had different initial traumata or none at all.

Consider the rats used in the present experiments. The control animals had occasional sores which came and went, but the sores which appeared in the deficient animals persisted. This was true for all wounds, including those which appeared for unknown reasons.

The urinary excretions of riboflavin correlated well with the amounts of riboflavin ingested. About 25% of the amount of riboflavin in a diet containing 1.6 mg per day is excreted. Since the 24-hour excretion drops markedly to approximately 10% when the intake of riboflavin is lowered to 1.1 mg per day, and remains at 10% for lower levels of intake, it can be suggested that the *basal* requirement for an adult is between 1.1 and 1.6 mg per day (Horwitt et al., '49). Emergency requirements during illness and for repair of tissue would necessarily be greater.

Three of the subjects, A1, A5 and A8, who had the most severe lesions had auburn beards. This may or may not have been a coincidence, but it is thought-provoking to add to this fact the observation that the one subject (BY3) in the previous project in this laboratory (Horwitt et al., '48, p. 55) who developed angular stomatitis is a distinct "red-head." For almost a year this man had been on a diet which provided 0.75 mg of riboflavin per day. The significance of the rather severe left oral lesion which spread over the left cheek was minimized because of a history of minor oral lesions which developed when the subject had been receiving riboflavin as a dietary supplement. This is another demonstration that such oral lesions per se are not necessarily due to riboflavin deficiency; but when they become worse and do not heal readily until after riboflavin is provided, then ariboflavinosis is a proper diagnosis.

The "yellow enzymes" are necessary in such a large number of cellular reactions (Sumner and Somers, '43) that it

is surprising that many tests of efficiency in addition to the flicker fusion test and psychological tests of mental fatigue have not produced positive results. The inability to find any changes in the metabolites in the body fluids may be because the cell, once matured, requires only a minimum amount of riboflavin. However, the rate of replacement of such cells with others may be dependent upon a more abundant supply of riboflavin, obtainable either from the diet or through the breakdown of less vital tissue.

SUMMARY

The effects of diets restricted in riboflavin but otherwise adequate were observed in 15 male subjects. Fourteen other subjects were observed simultaneously as controls. Depletion with respect to riboflavin on a diet of 0.55 mg in 2,200 cal. was maintained for from 9 to 17 months, during which period angular stomatitis, seborrheic dermatitis, scrotal skin lesions, and diminution of ability to perceive flicker were observed.

Simultaneous experiments in which the same diet was fed to rats confirmed the inadequacy of the diet. In these animals retardation of wound healing was conspicuous.

Studies of the excretion of riboflavin in the urine suggest that the riboflavin requirement of a resting adult is between 1.1 and 1.6 mg per day. A reserve of riboflavin cannot be maintained on levels of intake below 1.1 mg.

Since previous studies revealed few abnormalities when the intake of riboflavin was above 0.6 mg per day, it may be concluded that allowances below this amount are insufficient to support normal tissue repair. The type of abnormality then encountered will be dependent upon the trauma, irritation, infection or other injuries to which the tissues are subjected.

Changes in the blood levels of lactic and pyruvic acids were not obtained on diets low in riboflavin, as opposed to the marked changes in carbohydrate metabolism observed during thiamine deficiency.

ACKNOWLEDGMENTS

Thanks are due to the members of the research unit who made this work possible. The laboratory staff, Mr. William T. Sullivan, Mr. Otto Lobstein, Mr. Paul Riggs, Miss Charlotte Kaplan, Mr. Sidney Silberberg, Mr. Lazarus Astrachan and Mr. Kurt Schmeer; the psychologists, Dr. Phyllis Wittman, Miss Arlene Ringold, Mrs. Mary Brown, Miss Esther Rollins and Miss Thelma Johnson: the dieticians, Miss Gordon Sampson, and Mrs. Mary Meyers: the cooks, Mrs. Pearl Ratcliff, Mrs. Beulah Spencer and Mrs. Maythel Newell; the nursing assistant, Mrs. Edna Newtson; and the many attendants who assisted with the subjects, all made important contributions. Special acknowledgment is due Dr. I. Zapolsky, who joined the project during its final stage to assume the duties of Dr. O. W. Hills, who left to accept a fellowship at the Mayo Clinic.

LITERATURE CITED

BAHR, P. H. 1914 A report on researches in sprue in Ceylon. Cambridge University Press, London.

BERRYMAN, G. H., C. R. HENDERSON, N. G. WHEELER, R. C. COGSWELL, J. R. SPINELLA, W. E. GRUNDY, H. C. JOHNSON, M. E. WOOD, C. W. DENKO, T. E. FRIEDEMAN, S. C. HARRIS, A. C. IVY AND J. B. YOUMANS 1947 Effects in young men consuming restricted quantities of B-complex vitamins and protein, and changes associated with supplementation. Am. J. Physiol., *148:* 618.

BOSSE, M. D., AND A. E. AXELROD 1948 Wound healing in rats with biotin, pyridoxin or riboflavin deficiencies. Proc. Soc. Exp. Biol. Med., *67:* 418.

GOLDBERGER, J., AND W. F. TANNER 1925 A study of the pellagra preventative action of dried beans, casein, dried milk and brewers' yeast. Pub. Health Rep., *40:* 54.

HORWITT, M. K., C. C. HARVEY, AND E. LIEBERT 1949 Correlation of urinary excretion of riboflavin with dietary intake and symptoms of ariboflavinosis. (In preparation.)

HORWITT, M. K., AND O. KREISLER 1949 The determination of early thiamine-deficient states by estimation of blood lactic and pyruvic acids after glucose administration and exercise. J. Nutrition, *37:* 411.

HORWITT, M. K., E. LIEBERT, O. KREISLER AND P. WITTMAN 1948 Investigation of human requirements for B-complex vitamins. National Academy of Sciences, Washington, D. C. Bull. Nat. Res. Council, no. 116.

HORWITT, M. K., G. SAMPSON, O. W. HILLS AND D. L. STEINBERG 1949 Dietary management of a study of riboflavin requirements. J. Am. Diet. Assoc., *25:* 591.

KEYS, A., A. F. HENSCHEL, O. MICKELSEN, J. M. BROZEK AND J. H. CRAWFORD 1944 Physiological and biochemical function in normal young men on a diet restricted in riboflavin. J. Nutrition, *27:* 165.

LANDOR, J. V., AND R. A. PALLISTER 1935 Avitaminosis B_2. Trans. Roy. Soc. Med. Hyg., *19:* 121.

MOORE, D. G .F. 1930 Partial loss of central acuity of vision for reading and for distance in school children and its possible association with food deficiency. West Afric. Med. J., *4:* 46.

PAUL, H. E., M. F. PAUL, J. D. TAYLOR AND R. W. MARSTERS 1945 Biochemistry of wound healing. I. The thiamine content of healing tissue of skin wounds. Arch. Biochem., *7:* 231.

ROBERTS, E. C., AND E. E. SNELL 1946 An improved medium for microbiological assays with Lactobacillus casei. J. Biol. Chem., *163:* 499.

SCOTT, H. H. 1918 An investigation into an acute outbreak of central neuritis. Ann. Trop. Med. Parasit., *12:* 109.

SEBRELL, W. H., AND R. E. BUTLER 1938 Riboflavin deficiency in man. Pub. Health Rep., *53:* 2282.

SLATER, E. C., AND D. B. MORELL 1946 A modification of the fluorimetric method of determining riboflavin in biological materials. Biochem. J., *40:* 644.

STANNUS, H. S. 1913 Pellagra in Nyasaland (2nd communication). Trans. Royal Soc. Trop. Med. Hyg., *7:* 32.

STRONG, F. M., R. E. FEENEY, B. MOORE AND H. T. PARSONS 1941 The riboflavin content of blood and urine. J. Biol. Chem., *137:* 363.

SUMNER, J. B., AND G. F. SOMERS 1943 Chemistry and Methods of Enzymes. Academic Press, New York, N. Y.

WILLIAMS, R. D., H. L. MASON, P. L. CUSICK AND R. M. WILDER 1943 Observations on induced riboflavin deficiency and the riboflavin requirements of man. J. Nutrition, *25:* 361.

THE RELATIONSHIP OF CARBOHYDRATE METABOLISM TO PROTEIN METABOLISM

III. FURTHER OBSERVATIONS ON TIME OF CARBOHYDRATE INGESTION AS A FACTOR IN PROTEIN UTILIZATION BY THE ADULT RAT

H. N. MUNRO [1]

Division of Animal Nutrition, University of Illinois, Urbana

ONE FIGURE

(Received for publication July 25, 1949)

When extra carbohydrate or extra fat is superimposed on an adequate diet, N is retained by the body (Cuthbertson and Munro, '37). Larson and Chaikoff ('37) made the interesting observation that time of ingestion is a factor in the protein-sparing action of extra carbohydrate. In their experiments, dogs were brought into N equilibrium on a mixed diet fed once daily and were then given single feedings of additional carbohydrate. It was found that this extra carbohydrate induced N retention only when given within a 4-hour interval before or after the mixed meal. From these experiments it was concluded that proximity of the additional carbohydrate to the protein of the mixed meal was essential for the protein-sparing effect to take place.

In 1939, Cuthbertson and Munro extended the question of time relationships to the protein and carbohydrate contained in ordinary diets. Experiments were carried out with adequate

[1] This work was carried out during the tenure of a Rockefeller Fellowship administered by the British Medical Research Council. Present address: Biochemistry Department, The University, Glasgow, Scotland.

diets which could be separated into two portions, one containing all or almost all of the carbohydrate and some of the fat of the diet, the other containing all of the protein and the remainder of the dietary fat. Four human subjects were studied and each received a constant daily amount of one diet throughout the experiment. When the carbohydrate-fat and protein-fat portions of the diet were eaten separately, the urinary N output was about 2 gm greater than when the meals consisted of a mixture of these portions. An increased output of urinary N was also observed when a diet given to adult rats was separated into carbohydrate-fat and protein-fat meals (Cuthbertson, McCutcheon and Munro, '40). The adverse effect on protein metabolism produced by eating the carbohydrate-fat and protein-fat portions of a diet at different times was attributed to separation of the dietary protein and carbohydrate, and it was concluded that time of carbohydrate ingestion plays an important part in protein utilization under normal nutritional circumstances. This hypothesis seems of sufficient interest to justify more rigorous examination, with a view to finding out whether changes in time of carbohydrate ingestion can by themselves account for the alterations in N excretion observed in the separation experiments, or whether the simultaneous separation of some of the fat of the diet from the dietary protein plays a part. Further experiments have accordingly been carried out on adult rats, using an experimental design in which the ingestion of only one dietary factor was varied at a time. These experiments show that a change in the time of carbohydrate ingestion can by itself cause alterations in N output. The effect is rather transitory. Changing the time of fat ingestion does not result in any significant alteration in N balance. The evidence for these conclusions follows.

EXPERIMENTAL

Adult male rats of the Sprague-Dawley strain were used. The diets consisted of purified ingredients — vitamin-free

casein,[2] corn starch, glucose,[3] lard or corn oil,[4] vitamins, minerals and roughage (wood flock) — and provided daily about 400 mg N and some 1,000 to 1,200 cal. of metabolizable energy per square meter of body surface area (the latter was estimated using the formula of Lee and Clark, '29). The animals drank only distilled water.

The rats were fed twice daily, at 9 A.M. and at 5 P.M. The food was weighed with great care into non-spilling feeding dishes and was moistened with water. The morning meal always contained the vitamins, minerals and wood flock of the diet in the form of a supplement [5] which also provided 0.83 gm carbohydrate, 0.28 gm fat and 3 mg N. The evening feed contained all the protein (casein) of the diet. Preliminary experiments showed that casein fed alone is unpalatable but is eagerly consumed if made into a paste with $NaHCO_3$. Throughout these experiments the casein was therefore mixed with 0.15 gm $NaHCO_3$, whether or not there were other constituents in the meal. The carbohydrate and fat (except for the supplement mentioned above) were mobile constituents of the diet, being given with either the morning or the evening feeding, according to the requirements of the experiment. The feeding routine of a typical experiment (the first to be described) is shown in table 1. The total constituents of the diet were kept constant throughout the experiment, the only variable factor being time of carbohydrate ingestion. During the first period all of the dietary carbohydrate and fat was given in the morning feeding. In the second period, carbohydrate was transferred from

[2] Labco.

[3] Cerelose.

[4] Mazola.

[5] Two grams of the supplement contained 829 mg starch, 226 mg wood flock, 226 mg cod liver oil, 54 mg wheat germ oil, 169 mg sodium chloride, 167 mg dicalcium phosphate, 112 mg potassium citrate, 77 mg calcium carbonate, 36 mg dipotassium phosphate, 19 mg magnesium carbonate, 7.5 mg ferric citrate, 590 μg manganous sulfate, 80 μg copper sulfate, 40 μg potassium aluminum sulfate, 40 μg cobalt chloride, 20 μg potassium iodide, 20 μg zinc carbonate, 0.4 μg sodium fluoride, 72.4 mg Merck's dry mix (25% choline chloride), 1.8 mg inositol, 900 μg *p*-aminobenzoic acid, 360 μg calcium pantothenate, 180 μg nicotinic acid, 45 μg thiamine hydrochloride, 45 μg riboflavin and 45 μg pyridoxine hydrochloride.

the morning to the evening feeding. During the third period (not included in table 1), the carbohydrate was transferred back to the morning meal, as in period I. This experiment permits one to compare N balance when protein and carbohydrate are taken separately (periods I and III) with N balance when they are eaten together (period II). In other experiments of similar design, fat was the mobile constituent of the diet.

It was clearly important in experiments involving the separate eating of two dietary constituents that a reasonable interval should elapse between the completion of one meal and

TABLE 1

Diet used in experiment 1 to study the effect of feeding carbohydrate separately from protein (period I) and together with protein (period II)

	PERIOD I		PERIOD II	
	Morning feeding	Evening feeding	Morning feeding	Evening feeding
Vitamin-mineral-roughage supplement	2 gm	..	2 gm	..
Corn oil	2.5 ml	..	2.5 ml	..
Glucose	4 gm	..	..	4 gm
Casein	..	2.5 gm	..	2.5 gm
$NaHCO_3$	.	0.15 gm	..	0.15 gm

the start of another meal. To ensure this, the feeding dishes were inspected at intervals throughout the day and if, as happened on rare occasions, a rat could not be coaxed to clean up the morning feeding several hours before the evening meal, the residue was removed and given with the morning feeding on the following day. Rats consistently refusing food were taken off experiment. There was never any difficulty in persuading the rats to clean up the evening meal quickly, once it had been appreciated that casein fed alone must be partly brought into solution with mild alkali in order to make it palatable.

Throughout the experiments the rats were housed in metabolism cages of the type used in this laboratory in the deter-

mination of the biological value of proteins. During the first 7 days of each experiment the rats were fed the full amount of the diet but the urine and feces were discarded. After this preliminary period of adjustment to the diet, urine and feces were collected separately at 9 A.M. every day; urine was stored with the addition of strong HCl. In most experiments analyses were made of the total urinary and fecal collections of 4-day periods. Two experiments were exceptions: in experiment 1 daily urine collections were analyzed, and in experiment 7 estimations were made on urine and feces collected over 7-day periods. Iron oxide was employed as a fecal marker to indicate the beginning and end of periods.

Food, feces and urine were analyzed for total N by macro-Kjeldahl estimation; mercury was used as the catalyst and digestion of food and feces samples was continued for 4 hours after clearing of the solution. This period of digestion seems to be adequate. One sample of casein analyzed in this way was found to contain 15.64% of N on a moisture-, ash- and fat-free basis; this agrees reasonably well with the average figure of 15.73% obtained for casein by Chibnall, Rees and Williams ('43) after a critical investigation of different catalysts and periods of digestion. Urine samples were digested for one hour after clearing. The data have been treated statistically and probabilities of less than 0.05 have been considered as likely to be significant, while values less than 0.01 have been regarded as highly significant.

RESULTS

1. Is protein utilization affected by the presence of carbohydrate at the same meal?

Experiment 1. Four rats with an average initial weight of 373 gm were used. They received a diet containing 2.5 gm protein,, 2.5 gm fat and 4.8 gm carbohydrate, 4 gm of carbohydrate (glucose) being mobile. The changes in diet during the course of the experiment are shown in table 1, and the mean daily output of N in the urine is recorded in figure 1.

During the preliminary week of adjustment to the diet and for the first few days of urinary collection (period I), all of the carbohydrate of the diet was given in the morning feeding and was therefore eaten separately from the protein of the diet. The output of urinary N remained reasonably constant during this period. Then the 4 gm of mobile carbohydrate were

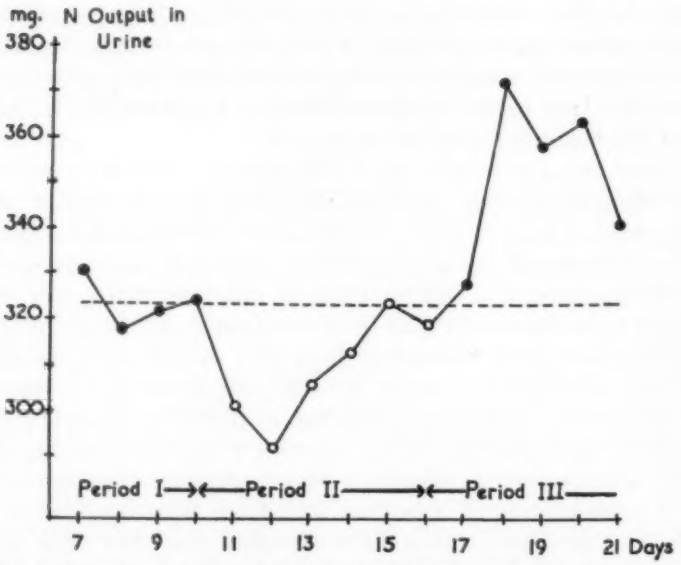


Fig. 1 Experiment 1. The effect of urinary output of altering the time of carbohydrate ingestion. Periods I and III, protein and carbohydrate eaten separately; period II, protein and carbohydrate eaten together.

removed from the morning feeding and given with the casein of the evening feeding (period II). There was at once a depression of N output which was, however, quite transitory, and after 4 days N output had regained levels similar to the average output of period I (shown in figure 1 by the dotted line). Then the mobile carbohydrate was once more given in the morning feeding (period III) and N excretion responded by increasing markedly; this effect was also of short duration,

and within 5 days N output had fallen nearly to the average level of period I.

During the experiment feces were collected and it was possible to compute the N balance of each rat for each period. In period I, when protein and carbohydrate were eaten separately, the mean daily N balance of the 4 rats was + 22.4 mg N; in period II, when protein and carbohydrate were taken at the same meal, it was + 41.5 mg; in period III, when protein and carbohydrate were again separated, the mean daily N balance was — 4.2 mg. Analysis of variance shows that these mean values differ from one another significantly (for the difference between periods I and II, P = 0.05–0.02; for the difference between periods I and III, P = 0.02–0.01; and for the difference between periods II and III, P is less than 0.01). A point of some interest is that, taking the mean daily N balance of period I as a base line, the extra N retained during the 6 days of period II amounted to 114.6 mg per rat, while the extra N excreted during the 5 days of period III was 133.0 mg. The fact that these figures are of the same order of magnitude suggests that the N retained when carbohydrate and protein were associated in period II was all eliminated again when the times of carbohydrate and protein ingestion were separated in period III.

Experiments 2, 3 and 4. Three confirmatory experiments [6] were carried out along the same lines as experiment 1, except for two points. First, only one dietary change was made, from carbohydrate and protein fed separately to carbohydrate and protein fed together. Secondly, a control group of rats received the diet without any change being made in the order of feeding during the experiment. In experiments 2 and 4 the diet was the same as that used in experiment 1; in experiment 3 the fat was reduced to 2.1 gm and the carbohydrate to 4.3 gm, and only 3.5 gm of glucose were transferred from the morning to the evening meal. Table 2 gives the mean daily N balances computed for 4-day periods beginning after the rats had been

[6] A further experiment along these lines had to be abandoned because the control group showed a significant improvement in N balance during period II.

TABLE 2

Experiments 2, 3 and 4. The effect of transferring carbohydrate from the morning (protein-free) meal to the evening (protein-containing) meal. (Experiment 4 also includes results from a group in which carbohydrate was added to the diet at the evening meal during period II)

EXPT. NO.	GROUP	NO. IN GROUP	MEAN INITIAL WEIGHT	DAILY N INTAKE	PERIOD I		PERIOD II		PERIOD III	
					Evening feeding	Mean daily N balance	Evening feeding	Mean daily N balance	Evening feeding	Mean daily N balance
			gm	*mg*		*mg*		*mg*		*mg*
2.	Carbohydrate transferred	4	314.5	369.2	Protein	+ 32.8	Protein and carbohydrate	+ 46.4	Protein and carbohydrate	+ 20.7
	Control	5	307.0	369.2	Protein	+ 34.0	Protein	+ 26.4	Protein	+ 27.6
3.	Carbohydrate transferred	4	311.1	373.3	Protein	+ 25.5	Protein and carbohydrate	+ 57.2	Protein and carbohydrate	+ 36.4
	Control	4	307.8	373.3	Protein	+ 29.0	Protein	+ 29.4	Protein	+ 29.5
4.	Carbohydrate transferred	3	326.5	374.3	Protein	+ 52.9	Protein and carbo hydrate	+ 92.6	..	..
	Carbohydrate added	3	328.0	374.3	Protein	+ 40.0	Protein and carbohydrate	+ 90.8	..	..
	Control	3	329.5	374.3	Protein	+ 41.8	Protein	+ 34.3	..	..

In experiment 2 the mean daily N balance of the experimental group during period II is significantly more positive than the mean N balance of the control group ($t = 3.18$; $P = 0.02$–0.01).

In experiment 3 the difference in mean daily N balances of the experimental and controls groups during periods II and III combined is significant ($t = 3.07$; $P = 0.05$–0.02).

In experiment 4, comparison of the N balance values obtained during period II after adjustment by co-variance for differences in N balance during period I shows that the values for the first two groups are very significantly different from the mean value for the control group ($P < 0.01$).

on the diet with carbohydrate and protein fed separately for one week. The time at which the mobile carbohydrate was fed during each period is indicated in the table by the composition of the evening meal.

All three experiments demonstrate that N balance improves when carbohydrate is transferred from the protein-free meal to the protein-containing meal without altering the total constituents of the diet. In each experiment the effect is statistically significant by comparison with the behavior of N balance in the corresponding control group. In experiments 2 and 3, carbohydrate and protein were fed together for two successive 4-day periods (periods II and III); in experiment 2 the beneficial effect on N balance was limited to period II and in experiment 3 it was mainly confined to period II. Thus the association of carbohydrate and protein at the same meal does not result in a permanent improvement in N balance. This finding is in agreement with the results of experiment 1.

Experiment 4 includes an additional feature. During period II the effect of transferring carbohydrate from the protein-free to the protein-containing meal was compared with the effect of *adding* the same amount of carbohydrate (4 gm glucose) to the diet along with the protein meal. The added carbohydrate had the same effect on N balance as the transferred carbohydrate, which indicates that the essential condition causing N retention is the association at the same meal of protein and carbohydrate. It should be added that N balance was studied in the second group for a further 8 days and it was found that the addition of supplementary carbohydrate to the evening meal resulted in a more prolonged improvement in N balance than could be achieved by simply transferring carbohydrate from the morning meal.

2. Is protein utilization affected by the presence of fat in the protein meal?

Experiments 5 and 6. In experiment 5, the effect of transferring fat from the protein-free meal to the protein-containing

meal was compared with the effect of a similar transfer of carbohydrate. In experiment 6 these transfers were carried out in the opposite direction. The diet in each experiment was the same as that used in experiment 1; 2.2 gm corn oil and 4 gm glucose were the mobile constituents.

In experiment 5 the rats were divided into three groups, each of which started by receiving all the dietary constituents except the protein in the morning meal. The mean daily N balances for the first 4-day period of collection (period I) are given in table 3. During the next 4 days of the experiment (period II), the first group were given the mobile fat in the evening meal. The second (control) group received the diet as fed during period I, and for the third group the mobile carbohydrate was transferred to the evening meal. Comparison of the mean N balances during periods I and II shows that the balances were slightly less favorable during period II in the case of the control group and the group receiving fat in the evening meal, but the group receiving carbohydrate with the protein of the evening meal had a significantly better N balance.

In experiment 6, in which the procedure was reversed, N balance was not affected by transferring fat from the evening to the morning meal, whereas N balance was adversely affected by a similar change in the time of carbohydrate ingestion (table 3). This difference in the behavior of the two groups is statistically highly significant.

Experiment 7. This comprises a series of three successive studies in which carbohydrate or fat or both were removed from a protein-containing meal. The diet supplied 2.8 gm protein, 2.3 gm fat and 6.5 gm carbohydrate. In each experiment the rats began by receiving 1.5 gm of the dietary fat (as lard) and 3 gm of carbohydrate (equal parts starch and glucose) in the evening meal with the protein of the diet. After the usual preliminary period, urine and feces were collected for 7 days (period I). During the next 7 days (period II) the three groups of rats received the diet arranged in different ways. In the first group, the fat was transferred from the evening meal to the morning meal, leaving the carbohydrate still in association

TABLE 3

Experiment 5. The effect of transferring fat compared with the effect of transferring carbohydrate from the morning (protein-free) to the evening (protein-containing) meal

Experiments 6 and 7. The effect of transferring fat or carbohydrate or both from the evening (protein-containing) to the morning (protein-free) meal

EXPT. NO.	GROUP	NO. IN GROUP	MEAN INITIAL WEIGHT	DAILY N INTAKE	PERIOD I		PERIOD II		DIFFERENCE IN MEAN DAILY N BALANCE		
					Evening feeding	Mean daily N balance	Evening feeding	Mean daily N balance	II–I	t	P
			gm	*mg*		*mg*		*mg*	*mg*		
5.	Fat transferred	8	317.7	371.7	Protein	+ 25.6	Protein and fat	+ 23.2	— 2.4	0.55	0.6–0.5
	Control	8	315.4	371.7	Protein	+ 36.9	Protein	+ 29.3	— 7.6		
	Carbohydrate transferred	7	319.6	371.7	Protein	+ 41.4	Protein and carbohydrate	+ 66.2	+ 24.8	3.94	< 0.01
6.	Fat transferred	3	336.3	374.3	Protein and fat	+ 29.9	Protein	+ 36.4	+ 6.5	6.44	< 0.01
	Carbohydrate transferred	3	338.8	374.3	Protein and carbohydrate	+ 39.2	Protein	+ 9.7	— 29.5		
7.	Fat transferred	4	463.5	409.8	Protein, fat and carbohydrate	+ 39.5	Protein and carbohydrate	+ 46.5	+ 7.1	0.73	0.6–0.5
	Carbohydrate transferred	4	466.5	412.8	Protein, fat and carbohydrate	+ 51.1	Protein and fat	+ 16.0	— 35.1	8.67	< 0.01
	Carbohydrate and fat transferred	4	446.3	415.7	Protein, fat and carbohydrate	+ 39.3	Protein	+ 28.3	— 10.9	6.42	< 0.01

with the protein. This change did not affect N balance. In the second group, the carbohydrate was transferred to the morning meal, leaving the fat with the protein of the evening meal; N balance was adversely affected by this separation of carbohydrate and protein. In the third group, all of the carbohydrate and fat in the diet was fed in the morning meal, leaving only protein in the evening meal during period II; N balance was again adversely affected. Thus those changes which affected the time of ingestion of carbohydrate caused highly significant alterations in N balance, whereas the one change which affected only the time of fat ingestion did not alter N balance.

3. Is protein utilization affected by fat given a short time before the protein meal?

Experiment 8. The failure of fat fed with protein to influence protein utilization might conceivably be due to a lack of coincidence in the times of fat and protein metabolism. There is no reason to believe that fat is more rapidly absorbed and metabolized than protein; the only possibility worth considering is whether it is too slowly utilized to spare protein. An experiment was therefore carried out in which fat was given some time before the protein meal and the effect on N balance examined. The diet used contained 2.5 gm protein, 2.1 gm fat and 4.3 gm carbohydrate; 1.8 gm fat (corn oil) and 3.5 gm carbohydrate (glucose) were the mobile constituents. The rats were divided into 4 groups. During the preliminary week of feeding and for the first 4-day period of urinary and fecal collection, all 4 groups received the whole of this diet except the protein in the morning meal. During this first period the different groups had mean daily N balances of similar magnitude (table 4). During the next 4-day period (period II) the groups received different dietary treatments. The first group were fed the diet as given in period I, and N balance remained unchanged. The second group of rats were given the mobile fat one and one-quarter hours before the evening (protein-containing) meal; N balance was not different from that of

TABLE 4

Experiment 8. The effect of removing fat from the morning (protein-free) meal and giving it shortly before the evening (protein-containing) meal

GROUP	NO. IN GROUP	MEAN INITIAL WEIGHT	DAILY N INTAKE	PERIOD I		PERIOD II		DIFFERENCE IN MEAN DAILY N BALANCE
				Evening feeding	Mean daily N balance	Evening feeding	Mean daily N balance	
		gm	*mg*		*mg*		*mg*	*mg*
Controls	4	307.8	373.3	Protein	+ 29.0	Protein	+ 29.4	+ 0.4
Fat given 1½ hrs. before	4	308.0	373.3	Protein	+ 20.4	Fat before protein	+ 19.0	— 1.4
Fat given 2½ hrs. before	4	307.3	373.3	Protein	+ 19.0	Fat before protein	+ 17.3	— 1.7
Carbohydrate transferred	4	311.1	373.3	Protein	+ 25.5	Protein and carbohydrate	+ 57.2	+ 31.7

period I. The third group received the fat two and one-half hours before the feeding of protein, and here also no improvement in N balance resulted. The rats in both of these groups consumed the mobile fat within 10 minutes of being offered it. The 4th group of rats were given the mobile carbohydrate in the evening meal during period II, and in this group the usual improvement in N balance was observed. Comparison of the mean N balances during period II after adjustment by covariance for differences in N balance during period I shows that the behavior of the 4th group is significantly different from the behavior of the other three groups ($P = 0.05–0.01$).

DISCUSSION

In experiments 1 through 4, the rats first received a diet with the protein given apart from the other constituents and then they received the same diet with most of the carbohydrate fed along with the protein. The immediate effect of this change in the time of feeding carbohydrate was a considerable improvement in N balance. This finding confirms the hypothesis proposed in earlier papers (Cuthbertson and Munro, '39; Cuthbertson, McCutcheon and Munro, '40), namely, that protein utilization is affected by the presence of carbohydrate in the same meal.

In our earlier experiments, attempts were made to determine the duration of the disturbance in protein metabolism caused by separating dietary protein and carbohydrate, but they were defeated by loss of appetite. The present experiments have not been complicated by this factor, probably because a more complete vitamin mixture was offered. Experiments 1 through 3 show that the disturbances in N balance produced by feeding carbohydrate together with protein are quite temporary, N output returning to its former levels within a few days of a change being made in the time of feeding the carbohydrate. This does not mean that thereafter carbohydrate fed with protein ceases to have any effect on protein metabolism; if the dietary carbohydrate and protein are separated again, there is an immediate loss of N roughly equivalent

to the amount retained when carbohydrate and protein were first taken together (fig. 1). This observation indicates that the continued feeding of carbohydrate with protein is necessary in order to prevent the retained material from being lost again.

One possible explanation of these phenomena is that they are due to variations in the amount of labile protein in the body, especially labile liver protein. It has been established that the amount of protein in the liver is considerably influenced by protein intake, and that the effect of changing from one dietary protein level to another is nearly complete within a few days (Addis, Poo and Lew, '36; Kosterlitz, '47). The improvement in protein utilization brought about by taking carbohydrate along with protein is presumably equivalent to an increase in protein intake and causes a rapid deposition of labile protein in the liver until an equilibrium between synthesis and catabolism has been reached. The attaining of this equilibrium is indicated by the return of N balance to normal levels. If the carbohydrate is then removed from the protein-containing meal, conditions for protein synthesis become less favorable and labile protein is discharged until a new equilibrium between synthesis and catabolism is attained. While it seems likely that changes in the amount of labile liver protein could account for the N balance phenomena of these experiments, one cannot exclude the possibility that other synthetic aspects of protein metabolism are favorably influenced by taking carbohydrate with protein. Cuthbertson, McCutcheon and Munro ('40) did not observe any appreciable difference in growth rate between rats receiving a diet separated into protein-fat and carbohydrate-fat portions and rats receiving the same diet in mixed form. Geiger ('48) has pointed out, however, that the growth rate in these experiments may have been limited by factors other than protein intake. Using a diet which supported better growth, he has been able to demonstrate a retardation of growth rate when protein and carbohydrate were separated by division of the diet into two portions. It would be desirable to study the question of growth

further, using an experimental design similar to the one described in the present paper, in which time of carbohydrate ingestion is the only variable.

Experiments 5 through 7 show quite clearly that no significant change in N balance occurs when the time of fat ingestion is altered to coincide with or differ from the time of taking protein. The possibility that the fat is too slowly metabolized to spare protein taken at the same meal is excluded by experiment 8, in which fat was taken one and one-quarter and two and one-half hours before the protein meal, without affecting N balance. Each of these experiments included a group of rats for which a corresponding change in the time of carbohydrate ingestion was made, and these animals showed significant alterations in N balance. Furthermore, the calories provided by the mobile fat were greater than those provided by the mobile carbohydrate in each of the experiments. We can therefore conclude that fatty acids do not act like carbohydrates in sparing protein given at the same meal. It is, of course, possible that the glycerol of the fat may exert the same effect as carbohydrate, but the amount present in the mobile fat of these experiments was too small to make such an effect likely to be detected. An additional conclusion from these experiments is that the giving of fat along with protein does not impair N balance. This excludes the possibility, inherent in our earlier experiments on man and the rat, that separation of diets into protein-fat and carbohydrate-fat portions may affect protein metabolism adversely because of the fat in the protein-fat portions.

SUMMARY

1. Adult male rats were first given a diet with the protein fed apart from the carbohydrate and then the same diet with most of the carbohydrate fed along with the protein. The immediate effect of this change in the time of feeding carbohydrate was a considerable improvement in N balance. After a few days, however, N balance returned to its original level. Subsequent separation of the times of ingestion of carbo-

hydrate and protein caused a transient impairment of N balance. It has been concluded that protein utilization is affected by the presence of carbohydrate in the same meal.

2. No change in N balance was observed in experiments involving similar alterations in the time of feeding fat. The giving of fat a short time before the protein meal was also without effect on N balance.

ACKNOWLEDGMENTS

The author appreciates greatly the courtesy of the University of Illinois in extending to him the use of its laboratories and other facilities for the investigations described in this project.

He is also grateful to Dr. R. A. Robb of the Statistical Department of Glasgow University for advice on the statistical treatment of the data.

LITERATURE CITED

ADDIS, T., L. J. POO AND W. LEW 1936 The rate of protein formation in the organs and tissues of the body. I. After casein refeeding. J. Biol. Chem., *116:* 343.

CHIBNALL, A. C., M. W. REES AND E. F. WILLIAMS 1943 The total nitrogen content of egg albumin and other proteins. Biochem. J., *37:* 354.

CUTHBERTSON, D. P., A. MCCUTCHEON AND H. N. MUNRO 1940 The relationship of carbohydrate metabolism to protein metabolism. II. Note on the effect of separation in time of the protein and carbohydrate moities of the diet of the adult and growing rat. Ibid., *34:* 1002.

CUTHBERTSON, D. P., AND H. N. MUNRO 1937 A study of the effect of overfeeding on the protein metabolism of man. III. The protein-saving effect of carbohydrate and fat when superimposed on a diet adequate for maintenance. Ibid., *31:* 694.

———— 1939 The relationship of carbohydrate metabolism to protein metabolism. I. The roles of total dietary carbohydrate and of surfeit carbohydrate in protein metabolism. Ibid., *33:* 128.

GEIGER, E. 1948 The importance of the time element in feeding of growing rats. experiments with delayed supplementation of protein. Science, *108:* 42.

KOSTERLITZ, H. W. 1947 The effects of changes in dietary protein on the composition and structure of the liver cell. J. Physiol., *106:* 194.

LARSON, P. S., AND I. L. CHAIKOFF 1937 The influence of carbohydrate on nitrogen metabolism in the normal nutritional state. J. Nutrition, *13:* 287.

LEE, M. O., WITH THE TECHNICAL ASSISTANCE OF E. CLARK 1929 Determination of the surface area of the white rat with its application to the expression of metabolic results. Am. J. Physiol., *89:* 24.

THE ROLE OF PYRIDOXINE IN ECOMOMY OF FOOD UTILIZATION [1]

BARNETT SURE AND LESLIE EASTERLING
Department of Agricultural Chemistry, University of Arkansas, Fayetteville

(Received for publication June 24, 1949)

In 1941 Sure and Dichek demonstrated that riboflavin produces a most pronounced effect on economy of food utilization, and that the increases in body gains are derived mainly from fats and to a lesser but appreciable extent from proteins.

EXPERIMENTAL

In the present communication the authors are reporting on the role of pyridoxine in economy of food utilization. Twenty-four male and 24 female albino rats were depleted of pyridoxine. The criteria for detecting symptoms of pyridoxine deficiency were dermatitis associated with alopecia and rough, sticky hairs (György and Eckardt, '39; Antopol and Unna, '42). We did not encounter any of the convulsive symptoms described by Chick et al. ('40) and by Daniel and associates ('42). In 26 to 59 days 15 rats, 10 males and 5 females, developed the typical dermatitis-alopecia symptoms and were used for this study, which from the start was conducted as a series of controlled feeding experiments. In each pair of animals the positive control, which received daily 25 μg of pyridoxine, was allowed the same amount of food consumed by its litter mate, which received no pyridoxine. The food

[1] Research paper no. 875, Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Experiment Station. An abstract of this paper was presented before the American Institute of Nutrition at Detroit, Michigan, April 20, 1949.

intake was balanced 4 times weekly. The animals at the beginning of the experiments were 26 to 30 days of age and each weighed 45 to 48 gm.

Cerecedo and Fay ('44) demonstrated a relationship between pyridoxine and protein. They found rats on a low protein, pyridoxine-deficient diet to be quite resistant to pyridoxine avitaminosis, and that on diets high in casein the acrodynia appeared at an earlier period and was more severe than on diets low in casein. We chose a diet of the following composition in parts per 100: casein,[2] 30; cellu flour, 2; salts no. 1 (Sure, '41), 4; vegetable shortening,[3] 5; cod liver oil, 3; and sucrose, 56. Each animal received daily 6 times weekly, separate from the ration, the following crystalline components of the vitamin B complex: 25 μg thiamine, 25 μg riboflavin, 25 μg niacin, 150 μg calcium pantothenate, 3 mg *p*-aminobenzoic acid, 6 mg choline chloride, and 1 mg inositol. The positive control rats received in addition 25 μg of pyridoxine daily.

Each pair of animals was sacrificed at the end of the vitamin depletion period (table 1, column 2) when the pyridoxine deficient rats developed the typical dermatitis-alopecia-sticky and rough hair symptoms and were analyzed for protein ($N \times 6.25$), fat, moisture, and ash according to the technique previously described (Sure and Dichek, '41). In addition, 24 male and 24 female rats of the same initial ages and weights were taken one week after weaning from stock diets and analyzed for protein, fat, moisture, and ash. From the chemical compositions and body weights at the beginning and at the end of the experimental periods, the chemical composition of body gains was computed. The results are summarized in table 1.

It is apparent from this table that while there are great variations among the different groups of animals in the period at which symptoms of pyridoxine deficiency began to be evident, and in the rates of growth of the avitaminotic and control animals, the increased gains in body weight per 100

[2] Smaco brand.

[3] Crisco.

gm of food intake of the litter mates which received 25 μg of pyridoxine daily are striking in every case. The gain in body weight per 100 gm of food intake, which expressed the re-

TABLE 1

Role of pyridoxine in economy of food utilization
(P = pathological; RC = restricted control)

PAIR NO.	PERIOD OF VITAMIN DEPLÆTION	CHANGES IN BODY WEIGHT	TOTAL FOOD INTAKE	GAIN IN WEIGHT PER 100 GM FOOD INTAKE	CHEMICAL COMPOSITION OF BODY GAINS		
					FAT	ASH	PROTEIN
	days	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
1 P ♂	26	+ 12	157.3	7.6	+ 0.98	+ 1.46	+ 2.50
RC ♂		+ 46	165.3	27.9	+ 4.15	+ 2.56	+ 10.68
2 P ♂	34	+ 17	236.2	7.2	— 3.20	+ 1.23	+ 3.81
RC ♂		+ 78	257.7	30.2	+ 6.30	+ 2.00	+ 16.90
3 P ♂	33	+ 26	227.6	11.4	— 2.14	+ 1.90	+ 6.22
RC ♂		+ 67	232.6	28.8	+ 8.10	+ 3.48	+ 15.10
4 P ♂	42	+ 18	272.8	6.6	— 0.06	+ 2.42	+ 6.82
RC ♂		+ 64	288.2	22.2	+ 10.43	+ 3.59	+ 15.08
5 P ♂	40	+ 50	298.3	16.8	— 1.70	+ 2.20	+ 11.58
RC ♂		+ 90	303.0	29.7	+ 11.18	+ 3.40	+ 17.62
6 P ♀	47	+ 23	295.0	7.8	— 0.32	+ 1.17	+ 6.41
RC ♀		+ 54	302.0	17.9	+ 11.15	+ 2.55	+ 11.65
7 P ♂	49	+ 42	338.4	12.4	— 2.44	+ 2.80	+ 11.81
RC ♂		+ 95	346.0	27.4	+ 7.54	+ 4.72	+ 25.60
8 P ♂	47	+ 33	275.6	12.0	— 2.70	+ 1.90	+ 7.89
RC ♂		+ 83	278.7	29.7	+ 3.99	+ 3.29	+ 15.80
9 P ♂	51	+ 23	300.0	7.7	— 3.70	+ 2.34	+ 9.58
RC ♂		+ 82	301.0	27.2	+ 7.31	+ 3.16	+ 16.57
10 P ♂	52	+ 62	381.4	16.3	+ 4.52	+ 3.29	+ 15.12
RC ♂		+ 98	381.2	32.6	+ 10.91	+ 3.42	+ 22.96
11 P ♂	51	+ 65	384.3	16.9	+ 1.24	+ 3.32	+ 12.03
RC ♂		+ 98	391.9	25.0	+ 7.76	+ 4.93	+ 23.07
12 P ♀	52	+ 43	366.3	11.7	— 0.48	+ 2.08	+ 10.47
RC ♀		+ 70	375.2	18.7	+ 12.17	+ 3.30	+ 15.97
13 P ♀	59	+ 60	431.3	13.9	+ 3.64	+ 2.71	+ 14.34
RC ♀		+ 89	427.1	20.8	+ 7.38	+ 3.88	+ 21.65
14 P ♀	54	+ 23	282.5	8.2	— 3.31	+ 1.80	+ 6.71
RC ♀		+ 55	278.4	19.8	+ 6.12	+ 2.52	+ 11.99
15 P ♀	63	+ 65	427.5	15.2	+ 6.09	+ 3.00	+ 14.90
RC ♀		+ 90	428.3	21.0	+ 7.36	+ 4.25	+ 21.43
Average P		37.5	325	11.4	— 0.24	+ 2.24	+ 9.34
RC		75.9	312	25.2	+ 8.12	+ 3.40	+ 17.47

quirements for maintenance and growth, was the yardstick we used for evaluating economy of food utilization.

Averaging the results for 15 paired feeding experiments on the same food intake, 25 μg of pyridoxine daily produced a 121% increase in economy of food utilization, an increase of over 800% in fat synthesis, an increase of 86% in protein synthesis, and an increase of 51% in the minerals in the animal body.

SUMMARY

Pyridoxine produces a marked effect on economy of food utilization. The increases in body gains are due largely to fat and protein synthesis and, to a less but appreciable extent, to increases of minerals in the animal body.

LITERATURE CITED

ANTOPOL, W., AND K. UNNA 1942 Lesions produced by diets free of vitamin B_6 (pyridoxine) and the response to vitamin B_6. Arch. Path., *33:* 241.

CERECEDO, L. R., AND J. R. FAY 1944 Protein intake and pyridoxine deficiency in the rat. Arch. Biochem., *5:* 207.

CHICK, H. M., M. ELSADER AND A. N. WORDEN 1940 Occurrence of fits of an epileptiform nature in rats maintained for a long period on a diet deprived of vitamin B_6. Biochem. J., *34:* 595.

DANIEL, E. P., O. L. KLINE AND C. D. TOLLE 1942 A convulsive syndrome in young rats associated with pyridoxine deficiency. J. Nutrition, *23:* 205.

GYÖRGY, P. AND R. E. ECKARDT 1939 Vitamin B_6 and skin lesions in rats. Nature, *144:* 512.

SURE, B. 1941 The existence of a new dietary factor essential for lactation. J. Nutrition, *22:* 499.

SURE, B., AND M. DICHEK 1941 Riboflavin as a factor in economy of food utilization. Ibid., *21:* 453.

A FATAL VITAMIN E DEFICIENCY DISEASE IN RATS CHARACTERIZED BY MASSIVE LUNG HEMORRHAGE AND LIVER NECROSIS [1]

E. L. HOVE, D. H. COPELAND AND W. D. SALMON
Laboratory of Animal Nutrition, Agricultural Experiment Station of the Alabama Polytechnic Institute, Auburn

SEVEN FIGURES

(Received for publication July 12, 1949)

Recent reports have ascribed to vitamin E an important role in preventing fatal liver necrosis in rats. Schwarz ('44) found that rats died with extreme liver damage after about 6 weeks on a diet containing 16% of alkali-treated casein. The liver damage and death were prevented by alpha-tocopherol, or by replacing the alkali-treated casein with casein purified by the usual methods. The damage to the liver appeared to be the massive hepatic necrosis first produced by Himsworth and Glynn ('44) as a syndrome distinct from diffuse hepatic fibrosis, and preventable by cystine or methionine.

György and Goldblatt ('49) noted that massive hepatic necrosis developed in about 40% of adult rats kept on a low casein diet when lard was included, but in later experiments less damage was seen when commercial hydrogenated cottonseed oil [2] was the fat source. This difference in results was

[1] Published with the approval of the Director of the Alabama Agricultural Experiment Station. A preliminary report of this work was given at the annual meeting of the Federation of American Societies for Experimental Biology, March 18–22, 1949. It was supported in part by a grant from the Nutrition Foundation, Inc. Acknowledgment is made to Merck and Company, Rahway, N. J., for gifts of B complex vitamins.

[2] Crisco.

attributed to the tocopherol content of the cottonseed oil. Direct experimental comparison of these results with the influence of tocopherol given rats on the low casein-lard diet was tried in one experiment by these investigators. The result, while suggestive, was difficult to evaluate since necrosis was seen in only three of the 18 rats not receiving tocopherol. In a second experiment with yeast as the source of protein, the tocopherol effect was clearly evident.

The observations of Schwarz ('44) relating to alkali-treated casein could not be duplicated by György and Goldblatt ('49), although an apparently identical experimental technique was used. Hove ('46a, '49) twice tried to repeat the findings of Schwarz, using both albino and hooded strains of rats, but was unsuccessful in both cases. Furthermore, Hove ('46b) reported the absence of gross liver damage in adult rats maintained for 20 weeks on a 5% casein, vitamin E-low diet, although alpha-tocopherol was dramatically effective in conserving body weight.

In view of the negative and variable results obtained previously, it came as a surprise to note in the experiments presently reported the death of a majority of the rats restricted to a 10% casein, vitamin E-low diet, while complete protection was given by vitamin E. Necrosis of the liver was seen in nearly all of these rats. Of great significance may be the observation that death was accompanied, and possibly caused, by massive lung hemorrhage.

EXPERIMENTAL

Diet and animals

The vitamin E-low diet (E-20) used for most of the work herein described had the following percentage composition: water-washed casein 10 (Salmon, '47), sucrose 56, salt mixture 4 (Salmon, '47), lard 19, cod liver oil U. S. P. 1, vitamin premix 10. The latter consisted of pure vitamins mixed with sucrose to supply the following levels per gram of diet: thiamine, riboflavin and pyridoxine, 5 μg each; calcium panto-

thenate 20 μg; niacin 40 μg; choline chloride 2 mg; *i*-inositol 0.2 mg; and menadione 2 μg.

In one of the experiments oxidized casein was used as the protein source. This was prepared by a method similar to that described by Toennies ('42) but differing in that 3% hydrogen peroxide was used instead of the recommended 30%. The more dilute hydrogen peroxide is equally effective in destroying methionine and tryptophan, and the reaction proceeds more smoothly and with less terminal foaming. The water-washed casein of the basal E-low diet was replaced by oxidized casein at a 16% level and the diet was supplemented with 0.3% of DL-tryptophan. The sucrose level was reduced in order to maintain all other ingredients at standard levels.

Over a period of a year, litters of hooded rats of the Alabama Experiment Station's high-choline-requiring strain were obtained from the stock colony at three weeks of age and at 40 to 50 gm in body weight. These were placed on the basal E-low diet (E-20), with about a third of each litter receiving 7 mg of dl-alpha-tocopherol weekly as an olive oil solution given orally. In more recent experiments dl-alpha-tocopherol acetate was added to the cod liver oil at a level to furnish 1 mg of vitamin E per 10 gm of diet. The experiments were continued until the death of the experimental animals, or for at least 100 days.

RESULTS

Description of the acute vitamin E deficiency

Over 75% of the male rats died after an average of 10 weeks on the vitamin E-low, 10% casein basal diet. No deaths occurred among the control rats receiving alpha-tocopherol (table 1). Female rats appeared to be much less sensitive to the lack of vitamin E, in that only 5 out of 15 females died on the basal E-low diet. These are not included in the averages given in table 1.

Prior to death the existence of a vitamin E deficiency was evidenced by the blanching of the incisor teeth and by the somewhat slower growth rate. In 5 cases in which the death

process was observed, the rats expired in convulsions. Aside from a few blood flecks about the nostrils, no external evidence of hemorrhage was seen. At necropsy a high incidence of massive lung hemorrhage and severe liver damage was noted. The subcutaneous blood vessels were very prominent. The bladder was distended in nearly every case and contained a large amount of loose sediment. The testes were somewhat smaller than those of the controls receiving tocopherol. The

TABLE 1

Fatal vitamin E deficiency among weanling male rats fed the 10% casein basal diet (E-20)

CATEGORY OF INTEREST	SUPPLEMENTS OF ALPHA TOCOPHEROL	
	None	7 mg weekly
Number of rats	51 [1]	32
Number of spontaneous deaths	37	0
Average number of days on diet	71	100
Liver (% of body weight)	5.0	4.2
Liver fat (crude ether ext., % fresh basis)	3.8	4.4
Testes (% of body weight)	1.7	1.9
Number of rats with gross liver damage	31	0
Number of rats with massive lung hemorrhage	28	0
Growth rate for 6 weeks (gm/rat/day)	0.9	1.3

[1] Six of these rats were removed from experiment between the 8th and the 12th weeks to determine whether the liver and lung lesions were progressive. The remaining non-fatalities are still on the diet after more than 250 days.

stomach, intestines, kidney, and spleen showed no grossly evident lesions. The abdominal cavity was free from excessive fluid.

As is illustrated in figure 1, the lungs were engorged with blood in 28 of the 37 fatal cases (table 1). This condition was extensive enough to be considered the cause of death in these rats. The heart was normal grossly except for some congestion of the coronary vessels, and in two cases scar tissue was evident. The large vessels appeared somewhat distended.

The liver was grossly damaged in 31 of the 37 fatal cases. The surface was smooth but showed a marble-like mosaic

caused by dark and light areas ranging up to 1 cm in width and involving all lobes. In extreme cases the surface of the light-colored areas was depressed. The weight of the liver relative to body weight was somewhat greater than that of the controls receiving vitamin E. The average fat content (fresh basis) of 6 damaged livers was 3.8%, while for the tocopherol-supplemented rats the liver fat was 4.4% (table 1). The moisture content was 69.1 and 69.8%, respectively.

The thymus gland was invariably rust colored, with small dark areas. The brain was normal in the gross. The posterior lobe of the pituitary was rust colored in a few cases.

Histological description of the lesions

The lung tissue of the rats that died due to acute vitamin E deficiency retained its normal architecture, but the interalveolar tissue was packed with red blood cells. There was marked distention of the large and small blood vessels. In some areas the alveoli were filled with red blood cells, as is shown in figure 2; for comparison, a section of lung from a control rat is shown in figure 3. There was no evidence of lung infection. The lack of organization of the red blood cell accumulations indicated that this condition was acute and could have been the cause of death in the animal. The peripheral subcutaneous blood vessels were also distended, as is shown in figure 1.

Microscopic studies of the slides prepared from the livers of the vitamin E-low rats revealed an extensive necrosis of centrilobular origin. The nature and extent of the necrosis is shown in figure 5. In most cases this type of necrosis occurred extensively in all lobes of the liver. In less widespread cases the damage was confined to smaller areas of certain lobes, and some lobes were fairly normal in appearance. In 7 cases entire lobes of the liver seemed to have undergone massive necrosis (fig. 6), and other lobes of the same liver showed patchy centrilobular necrosis with large areas of normal tissue. In three of the experimental animals large areas of the liver parenchyma were completely de-

stroyed and in some areas regeneration had occurred, which resulted in changes in the architecture of the liver, as is shown in figure 7. The livers of the control rats receiving vitamin E were normal (fig. 4). There was no indication of significant fat accumulations in these livers. No significant pathologic changes were observed in microscopic examination of tissues taken from other organs.

TABLE 2

The relation of various supplements to the fatal vitamin E deficiency disease

VARIATION IN LOW CASEIN DIET	NUMBER OF:		TIME OF DEATH	GROWTH RATE IN 6 WKS.
	Rats	Deaths		
			days	*gm/day*
Added casein (to 16%)	20	0	..	3.9
DL-methionine, 0.1%	5	4	58	1.3
L-cystine, 0.1%	5	2	85	1.6
Theophylline, 0.1%	5	5	54	0.7
Low calcium [1]	4	4	63	0.1
Low potassium [1]	4	4	58	0
High calcium [2]	4	2	86	0.2
High potassium [2]	4	4	65	0.5
None	4	3	67	0.8

[1] Salts of these elements were omitted from the diet.

[2] The carbonates of these elements were added to the diet at the expense of sucrose to double the normal supply.

The relation of protein to the fatal vitamin E deficiency

When the casein in the vitamin E-low diet was increased to 16% at the expense of the sucrose, all other ingredients remaining constant, death and the hemorrhagic syndrome did not occur. A large group of rats have been maintained for 15 months on such a diet, and the only benefit resulting from the vitamin E supplement given to the controls has been the maintenance of normal tooth color, better muscle tone, and greater maximum body weight.

Little if any benefit was derived from the addition to the low casein diet of L-cystine or DL-methionine at levels of 0.1%. The data shown in table 2 indicate that death with

typical symptoms ensued, although the severity of the condition may have been lessened somewhat by the cystine addition. It may also be noted in table 2 that theophylline was completely inactive. Neither the omission nor the doubling of the calcium or potassium content of the diet affected the course of the fatal vitamin E deficiency (table 2).

Data confirming the non-effectiveness of DL-methionine in preventing the fatal vitamin E deficiency are given in table 3. On a diet containing 16% of oxidized casein and supplemented with 0.3% DL-tryptophan, young rats lost weight

TABLE 3

The occurrence of the fatal vitamin E deficiency on a 16% oxidized casein diet supplemented with methionine and 0.3% DL-tryptophan

DL-METHIONINE ADDED TO DIET	ALPHA TOCOPHEROL SUPPLEMENT					
	None			7 mg weekly		
	Number of rats	Number of deaths	Growth rate (6 wks.)	Number of male rats	Number of deaths	Growth rate (6 wks.)
%			*gm/day*			*gm/day*
0	3	3 [1]	—0.6	3	3 [1]	—0.7
0.25	6	3	0	4	0	0
0.50	3	3	0.9	3	0	1.3
1.00	3	2	1.6	3	0	2.0

[1] The rats in these groups were all dead by the 5th week.

rapidly and died in 4 to 5 weeks. Vitamin E supplements did not influence this course. None of these rats showed gross pathological lesions of the liver, lungs, or other organs. With 0.25% of DL-methionine added to the diet, body weight was maintained but no growth occurred. Three of the 6 rats without vitamin E died before the 10th week on this diet; typical lung and liver lesions were present. With 0.5% of methionine added to the diet, growth was considerably better, especially with supplements of alpha-tocopherol; but all rats without vitamin E died with typical vitamin E deficiency lesions between the 8th and 10th weeks. With 1.0% of methionine added to the diet, growth was even better; but death

still occurred among the vitamin E-free group. It is to be noted that this diet contained no cystine.

The effect of the dietary fat level on the fatal vitamin E deficiency

The basal diet used in the preceding work contained 20% fat. To determine the effect of the dietary fat level on the incidence and severity of the fatal vitamin E deficiency, an experiment was set up using diets with fat levels ranging from 5% to 40%. Groups of 4 weanling male rats were placed on each of these diets. The results, as given in table

TABLE 4

The relation of the fat content of the 10% casein diet to the fatal vitamin E deficiency in weanling male rats

DIETARY FAT [1]	NUMBER OF:		AVERAGE TIME OF DEATH	AVERAGE GROWTH RATE IN 4 WKS.
	Rats	Deaths		
%			*days*	*gm/day*
5	4	2	59	1.5
10	4	3	53	1.3
20	4	3	62	1.1
40	4	4	58	0.9

[1] The fat in the diet consisted of 1% cod liver oil with the remainder lard.

4, show that the fat level in the diet did not have a profound influence on the incidence and severity of the fatal vitamin E deficiency. The casein levels of the diets were all kept at 10% and not balanced isocalorically with the fat level; this may account for the higher growth rate at the lower fat levels.

DISCUSSION

The development of fatal vitamin E deficiency in rats depends upon the simultaneous deficiency of some substance present in casein. Schwarz ('44) removed the casein co-factor by an alkali extraction, and though this technique has been unsuccessful in this laboratory, similar results have been obtained by using a low (10%) dietary level

of water-washed casein. Cystine and methionine appear not to be the sole factors present in casein. However, it should be emphasized that supplements of cystine or methionine higher than 0.1% in the 10% casein diet were not tried; at this level cystine appeared to give some protection, but methionine was inactive. Using higher levels, György and Goldblatt ('49) noted partial protection by either cystine or methionine, while Himsworth and Glynn ('44) obtained complete protection with cystine added to a basal diet containing traces of alpha-tocopherol furnished by peanut oil. In previous work Hove ('48) noted that methionine added at a 0.1% level to a diet of the same composition as that used in the present work was able to replace alpha-tocopherol for the protection of rats against death due to an acute CCl_4 toxicity. Theophylline was also a good protector against CCl_4, but in the present work, at the same dietary level, it was of no benefit. Schwarz ('44) obtained no protection against the fatal liver damage from either cystine or methionine but he did observe that xanthine could replace alpha-tocopherol in this function.

The precise interrelation of the sulfur amino acids, purine bodies, and alpha-tocopherol as they relate to liver damage, lung hemorrhage, and organic solvent toxicity is confused at present. A considerable amount of further study is required. The question may be raised of whether rats restricted to a 10% casein basal diet have, in fact, developed a true vitamin E deficiency. The multiplicity of symptoms ascribed to a lack of vitamin E in various species makes this difficult to answer; but if blanching of the incisor teeth and a slight retardation in the growth rate are accepted as valid criteria, then a vitamin E deficiency can be said to develop within the first few weeks on the diet. This has been observed previously by Hove and Harris ('47).

The ultimate cause of death in the fatal vitamin E deficiency is unknown. Either the massive lung hemorrhage or the liver necrosis may have been sufficient to produce death. The absence of repair processes and fibrosis in the hepatic architec-

ture except in a few cases, and the normal nature of the lungs of animals killed for examination, indicate that the reaction progresses very rapidly. A disorder of vascular origin that limits the blood supply to the liver causes liver necrosis, as has been noted by Himsworth ('47). The occurrence of a massive lung hemorrhage also emphasizes the possibility of a primary vascular disorder.

Mason ('43) has investigated the cause of death of the fetus in the vitamin E-deficient rat. His description is as follows: "Prior to exitus there appear abnormalities in the vascular system characterized by variable degrees of dilation and thrombosis of peripheral and deeper vascular channels and by either localized or diffuse areas of hemorrhage . . . ." This description fits equally well the pathology noted in rats dying of the fatal E deficiency, and supports the conclusion that the basic function of vitamin E is the same whether in preventing the death of young rats *in utero* or in protecting young growing rats against the fatal deficiency, when they are conditioned by a low casein intake.

SUMMARY

1. Young rats died of an acute vitamin E deficiency when restricted to a 10% casein diet. Death occurred suddenly in over 75% of the male rats between the 6th and 12th week, or after an average of 71 days on the diet.

2. Death was characterized by massive lung hemorrhage, centrilobular or massive necrosis of the liver, and distention of the subcutaneous blood vessels.

3. Protection against the fatal disease was given by alpha-tocopherol or by increasing the casein to a level of 16% in the diet. The disease occurred when the casein in the diet was replaced with 16% oxidized casein plus 0.3% tryptophan and up to 1% methionine. Cystine was slightly protective, but neither methionine nor theophylline was of benefit when included in the 10% casein diet at a 0.1% level.

LITERATURE CITED

GYÖRGY, P., AND H. GOLDBLATT 1949 Further observations on the production and prevention of dietary hepatic injury in rats. J. Exp. Med., *89:* 245–268.

HIMSWORTH, H. P. 1947 The Liver and Its Diseases. Blackwell Scientific Publications, London, p. 37.

HIMSWORTH, H. P., AND L. E. GLYNN 1944 Massive hepatic necrosis and diffuse hepatic fibrosis: their production by means of diet. Clin. Sci., *5:* 93–123.

HOVE, E. L. 1946a Unpublished data. Distillation Products, Inc., Rochester, N. Y.

———— 1946b Interrelation between alpha-tocopherol and protein metabolism: Body weight and tooth pigmentation of rats. Proc. Soc. Exp. Biol. Med., *63:* 508–511.

———— 1948 Interrelation between alpha-tocopherol and protein metabolism. III. The protective effect of vitamin E against CCl_4 poisoning in rats. Arch. Biochem., *17:* 467–474.

———— 1949 Unpublished data.

HOVE, E. L., AND P. L. HARRIS 1947 Interrelations between alpha-tocopherol and protein metabolism. II. The increased utilization of casein produced by tocopherol, yeast digest, or xanthine in the rat-growth protein quality test. J. Nutrition, *34:* 571–580.

MASON, K. E. 1943 A hemorrhagic state in the vitamin E deficient fetus of the rat. Essays in Biology. University of California Press, Berkeley, Calif., pp. 401–409.

SALMON, W. D. 1947 Some physiological relationships of protein, fat, choline, methionine, cystine, niacin and tryptophane. J. Nutrition, *33:* 155–168.

SCHWARTZ, K. 1944 Tocopherol als Leberschutzstoff. Zschr. physiol. Chem., *281:* 109–116.

TOENNIES, G. 1942 Oxidative conversion of casein into protein free of methionine and tryptophan. J. Biol. Chem., *145:* 667–671.

PLATE 1

EXPLANATION OF FIGURES

1 Gross dissection showing the massive lung hemorrhage, distended subcutaneous blood vessels, and blanched teeth of a vitamin E-deficient rat (left) which died after 10 weeks on Diet E-20, as compared to the normal appearance of a litter-mate control receiving vitamin E (right).

2 Photomicrograph of the lung of a vitamin E-deficient rat, showing extensive accumulation of red blood cells in the alveolar spaces and interalveolar tissue. Hematoxylin and eosin stain, × 470.

3 Photomicrograph of lung of control rat showing normal histology. Hematoxylin and eosin, × 230.

PLATE 1

PLATE 2

EXPLANATION OF FIGURES

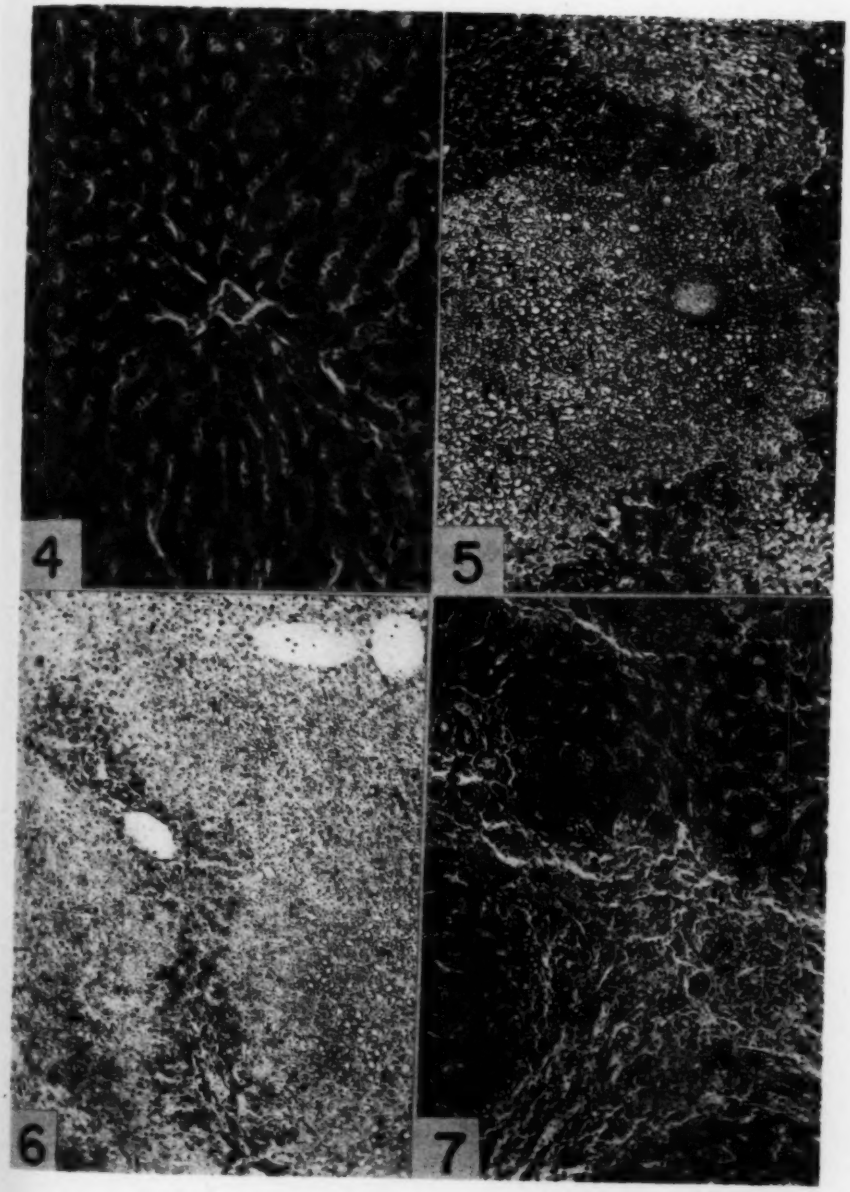
4 Photomicrograph of normal liver of control rat receiving vitamin E. Hematoxylin and eosin stain, × 180.

5 Photomicrograph showing centrilobular necrosis in liver of a vitamin E-deficient rat. This condition appeared in 31 cases. Hematoxylin and eosin, × 90.

6 Photomicrograph showing massive necrosis in the liver of an E-deficient rat. This condition appeared in 7 cases. Hematoxylin and eosin, × 90.

7 Photomicrograph of an area of the liver of a vitamin E-deficient rat, showing regeneration of liver and changes in architecture. This condition appeared in three cases. Hematoxylin and eosin, × 110.

PLATE 2



THE NUTRITIONAL EFFECTS OF HEAT ON FOOD PROTEINS, WITH PARTICULAR REFERENCE TO COMMERCIAL PROCESSING AND HOME COOKING

H. H. MITCHELL, T. S. HAMILTON AND JESSIE R. BEADLES
Division of Animal Nutrition, University of Illinois, Urbana

(Received for publication July 25, 1949)

The protein content of a food is not necessarily a good index of its value in protein nutrition. Its digestibility must be considered in a complete nutritional appraisal. Protein digestibility depends not only on the chemical nature of the protein (nitrogen) component of the food, but also upon the other constituents that may modify the action of digestive enzymes, by specific inhibition of an enzyme system, by interposing indigestible barriers between protease and substrate, by disturbing the proper relation between the secretory and motor activities of the gastrointestinal tract, or by other means. The exent of the utilization of the end-products of protein digestion, the amino acids, in metabolism is another important item in the nutritional appraisal of proteins, depending largely, though not entirely, on the proportions existing among those amino acids that are indispensable constituents of the diet.

Considerable information has been accumulated concerning the utilization of food protein (nitrogen) in digestion and in metabolism, using various species of animals as experimental subjects; much further information of a comparative nature has been secured by the use of experimental methods that do not distinguish between wastages of food nitrogen in digestion

and in metabolism. The preparation of a food for consumption by animal or man, by commercial processing or culinary practices, has been repeatedly shown to modify the completeness of utilization of protein in digestion or in metabolism, or in both. The effect may be favorable but is generally unfavorable, while the most effective agent in whatever effect is produced is heat, modified by the amount of water present in the food. Obviously the effect of heat will depend upon the temperature attained within the food and the time of exposure. There is still much to be learned concerning the effect of heat processing on the nutritive value of food proteins, which is merely one phase of the much larger problem of the total effect of heat processing on foods, including in particular its effect on the contained vitamins.

This report will be concerned with: (1) the changes in the nutritive value of the proteins of peanut meal, cottonseed flour, linseed meal and corn brought about by their subjection to commercial processes commonly used in their preparation for animal or human use; (2) the changes induced in the proteins of sunflower seed flour by autoclaving in the laboratory for 30 min. at 20 lb. steam pressure; and (3) the changes in the protein value of beef round brought about by roasting according to a standard method.

Total heat damage ranging from 10 to 20% in the net utilization of protein by the growing rat was demonstrated for the proteins of the peanut, corn, sunflower seed, and cottonseed, generally in protein digestibility rather than in biological value. Beef proteins were not appreciably injured by roasting, while the proteins of flaxseed were improved slightly in nutritive value, mainly because of an improvement in digestibility.

EXPERIMENTAL PROCEDURES

The heated and unheated, or very mildly heated, food products were compared with respect to the nutritional value of their content of conventional protein ($N \times 6.25$) by nitrogen metabolism studies with young albino rats weighing initially about 50 gm. The experiments were carried out by a method

developed in this laboratory (Mitchell, '24; '44) leading to the computation of coefficients of true digestibility, making due allowance for the metabolic fecal nitrogen, and of biological values, expressing the percentage of the absorbed nitrogen used for the replacement of endogenous losses and for growth. Generally an experimental group consisted of 5 pairs of rats, paired for equality of sex and litter membership and approximate equality in initial body weight. Throughout the three experimental periods, pair mates received the same amount of their respective diets in a reversal system of feeding whereby each rat in any one experiment received samples of both the unheated and heated product in different experimental periods, 5 rats in one order and the other 5 in the reverse order. The heated and unheated products were tested in periods 1 and 3, while in period 2 all rats received the standardizing diet containing 4% of whole egg protein; in this period the ratio of metabolic fecal N to air-dried food, and the ratio of minimum endogenous urinary N to the three-fourths power of the body weight, were determined. An experimental period lasted from 11 to 14 days, with collections of feces and urine during the last 7 days. Fe_2O_3 was used as a feces marker.

The experimental rations contained: a complete salt mixture 4%, NaCl 1%, wheat germ oil 0.5%, cod liver oil 1.5%, and the test food to provide 10% of protein, or as large an amount as possible, with lard added to raise the fat content to 12%, wood flock to raise the fiber content to 2%, and glucose, starch and sucrose to make up to 100%.

The effect of heat processing on peanut proteins

One thousand pounds of No. 2 Spanish peanuts were treated by the Anderson expeller process at an oil extraction mill in Enterprise, Ala. The peanuts were shelled, ground and heated to 246°F. before entering the expeller. The run in the expeller lasted 8 hours and 6 samples for biological assay were taken during the last 4 hours of the run. The temperature of the barrel at the discharge end was 210°F.

In addition, a sample of the same kind of peanuts from the same source was shelled, ground, and extracted with ethylene dichloride at the plant of the VioBin Corporation, at Monticello, Ill., at temperatures ranging from 75° to 80°C.

The samples will be referred to as No. 0, the solvent extracted sample, and Nos. 1 to 6, the successive samples taken from the Anderson expeller. In preliminary paired-feeding tests, samples 0 and 4, 1 and 5, 2 and 6, 0 and 2, 1 and 4, and 3 and 6 were compared by a method of feeding for equal gains and of diluting the better ration with a nitrogen-free mixture; this method has been described by Mitchell, Burroughs and Beadles ('36). At the end of each experiment in which this technique is employed the two rats in each pair have gained the same in body weight through a feeding period of 5 to 7 weeks on the same caloric intake but on different intakes of protein if the proteins under test differ appreciably in nutritive value.

Only in the comparison of samples 0 and 4 and 2 and 6 were the results significantly different, favoring the lower numbered sample. In the first of these cases the average grams gain in body weight per gram of protein consumed were 1.46 and 1.31, respectively, with a probability of 0.021 (Student, '08) that an average difference as great or greater than this (and in the same direction) would have been brought about by a random combination of the uncontrolled factors in the experiment. In the second case the average protein efficiency ratios were 1.33 and 1.16 in favor of sample 2, the probability being 0.0020 that uncontrolled factors acting at random would have produced a difference as great or greater than this favoring the 2 sample.

These paired-feeding results indicate that heat damage to the peanut proteins had ocurred to some small extent in the first samples removed from the expeller, but that this damage had not been aggravated by continued exposure to the expeller temperature up to a limit of 8 hours. It may be pointed out that results of weight gain experiments, even with controlled feeding, cannot be considered conclusive evidence of

the differential nutritive effects of proteins; first, because of possible differential activity of the animals on the two comparative rations, and, second, because of possible differences in the protein content of the gains.

Nitrogen metabolism studies were carried out in a comparison of the solvent-extracted peanut meal, No. 0, and peanut meal No. 1. The average results, with their statistical analysis, are given in the first line of table 1. Evidently the heat incident to the processing of sample No. 1 definitely depressed the biological value of the protein; there is also a suggestion that the digestibility of the protein was slightly increased. The total effect of the heat treatment, expressed as the percentage change in the net utilization of the protein (coefficent of digestibility multiplied by the biological value and divided by 100), was a depression in protein utilization of 5.4% ($P \pm 0.0037$).

A comparison by this method of peanut meals 3 and 6 failed to reveal any difference either in the digestibility of the proteins or in their biological values, although the biological values obtained for both samples, averaging 49.8 for meal 3 and 50.5 for meal 6, were distinctly lower than the average biological value for meal 1, 56.8. The average digestibility of the protein in meal 3 was 94.0, and that in meal 6, 93.2. The average net utilization of protein in the pooled data for peanut meals 3 and 6 was 46.9, compared with an average of 57.7 for the solvent-extracted meal. If these averages may be considered directly comparable, they reveal an average difference in heat damage to peanut proteins of 18.7% between meals 0 and 3.

Mitchell, Burroughs and Beadles ('36) studied the change in protein utilization in peanuts during commercial roasting, when the peanuts were heated to between 400 and 450°F. for 30 to 35 min. The average coefficients of true digestibility of protein for raw and roasted peanuts were 97.4 and 96.1, respectively, and the average biological values of the absorbed protein were 59.4 and 55.1, respectively. Applying Student's ('08) method for the analysis of paired differences, the mean dif-

TABLE 1

The results of the nitrogen metabolism studies
(Each value is the average of 10 determinations, with the exception noted)

PROTEIN SOURCE	UNHEATED MATERIAL		HEATED MATERIAL		SIGNIFICANCE OF AV. DIFFERENCE IN NET PROTEIN UTILIZATION DUE TO HEAT [1]		TOTAL HEAT DAMAGE TO PROTEIN	
	Digestibility	Biological value	Digestibility	Biological value	Digestibility	Biological value	Total	Significance
					P [2] =	P [2] =	%	P [2] =
Peanut meal [3]	94.8	60.8	95.7	56.8	0.08	0.00004	5.4 [9]	0.0037
Sunflower seed meal [4]	93.8	57.1	91.3	52.0	0.0015	0.0039	10.2	0.0046
Cottonseed flour [5]	89.7	61.5	84.6	58.1	< 0.001	0.0034	10.7	0.00004
Linseed meal [6]	84.8	69.2	87.5	72.2	0.022	0.060	— 6.6	0.031
Corn [7]	90.0	59.6	75.8	56.4	< 0.00001	0.055	20.1	0.00044
Beef round [8]	99.6	73.8	99.3	74.8	insignificant		— 1.0	0.27

[1] Net protein utilization is the product of the coefficient of true digestibility and the biological value divided by 100.
[2] The probabilities in this table are computed according to Student ('08).
[3] Solvent-extracted peanut meal versus "expeller" sample 1.
[4] Solvent-extracted sunflower seed meal versus autoclaved meal.
[5] Solvent-extracted cottonseed flour versus meal prepared by the hydraulic process.
[6] Solvent-extracted linseed meal versus expeller-processed meal. Each value in this test is an average for 5 rats.
[7] Unprocessed corn versus flaked corn.
[8] Raw versus roasted beef.
[9] See the text for further data on extent of heat damage to peanut proteins.

ference in digestibility is highly significant ($P = 0.019$), as is the difference in biological value ($P = 0.0023$).[1] Computing the total heat damage in this 1936 experiment as was done in the experiment reported in the preceding paragraphs, one obtains a value of 8.3%.

The greater heat damage to the proteins of the peanut in the expeller oil extraction process (18.7%) than in the roasting process (8.3%) is evidently due to the much more prolonged application of heat in the former case.

Buss and Goddard ('48), using the method of protein evaluation proposed by Osborne, Mendel and Ferry ('19), have reported the growth-promoting value of the protein of raw peanuts, peanuts boiled in water for 40 min., peanuts roasted at 320°F. for 40 min., and peanuts roasted at 356°F. for 40 min. The average protein efficiency ratios (grams gain in 8 weeks per gram of protein consumed, with diets containing 10% of protein) were, respectively, 1.74, 1.84, 1. 65 and 0.21. No statistically significant differences in growth-promoting value among the first three preparations were established by the assay method used. Lysine supplementation of the peanuts roasted at the higher temperature raised the protein efficiency ratio from 0.21 to 0.93.

The effect of autoclaving on sunflower seed proteins

Solvent-extracted sunflower seed meal was compared, with reference to the nutritive value of the proteins, with the same meal autoclaved at 20 lb. steam pressure for 30 min. The average results, with their statistical analysis, are given on the second line of table 1. This heat treatment significantly depressed both the digestibility of the protein and its biological value, with a total heat damage of 10.2% computed from the drop in net utilization.

[1] In analyzing the data on the biological value of the protein of raw peanuts, the aberrant value of 44 was omitted after application of Chauvenet's criterion. In the paired comparisons the value for the same rat for heated peanuts, 62, was also disregarded.

The effect of heat processing on the proteins of cottonseed flour

Two cottonseed flours were prepared from the same batch of cottonseed. One was defatted by the hydraulic process, where the decorticated seed is crushed and passed through a series of steam cookers in which the temperature increases progressively from 190 to 240°F. This process takes almost two hours, after which the material is put through a hydraulic press. The other flour was prepared by solvent extraction with ethylene dichloride at a low temperature. The average results of the nitrogen balance studies, with a statistical analysis of the differences between the solvent-processed flour ("unheated") and the hydraulic-processed flour ("heated"), are given in the third line of table 1.

The heat employed in hydraulic processing definitely lowered the digestibility of the protein in cottonseed flour as well as its biological value; the total heat damage, based on the decrease in the net utilization of the protein, averaged 10.7%.

Olcott and Fontaine ('41) have also reported a depression in the nutritive value of cottonseed proteins, in proportion to the time of heat treatment, induced by autoclaving at 17 lb. steam pressure (254°F.). Since the results were obtained by the Osborne, Mendel and Ferry ('19) method, they cannot be compared quantitatively with those contained in table 1.

The effect of heat processing on the proteins of linseed meal

The proteins of linseed meal were studied by a comparison of two products prepared from the same batch of flaxseed. One product was solvent-extracted at a low temperature with ethylene dichloride, while the other was treated for oil removal by the expeller process. The average results are given in the 4th line of table 1.

With linseed meal, heat improved the digestibility of the proteins ($P = 0.022$), and quite probably their biological value

as well (P = 0.060). The total improvement averaged 6.6%, with P equaling 0.031. In this test each value in the table is the average for only 5, instead of 10, rats. The analysis was effected by the computation of Fisher's ('44) "t" value, dividing the P value by two to put it on the same basis as a Student probability, which always relates to a difference between means in the same direction as that observed.

The effect of heat processing on the proteins of corn

In order to test the effect on the nutritive value of the proteins of corn of the processes involved in the manufacture of corn flakes, we secured samples of the unprocessed corn, of the flaked corn and of the final toasted product. In the preparation of these corn flakes, the corn was cut, water was added, and the corn was cooked for from 20 to 45 min. at a steam pressure of 45 to 50 lb. The final toasting process raised the rolled flakes to a temperature of 350 to 400°F. for a period of three to 4 min.

These corn flakes are used mainly in the preparation of dog food; they are not degerminated, as are the corn flakes used for human consumption, although the processing seems to be much the same (Jacobs, '44).

The results of comparing the unprocessed corn with the corn flakes prior to the toasting process (line 5 of table 1) reveal a marked and significant drop in the average digestibility of the protein, from 90.0 to 75.8%, and a smaller drop in the average biological value, from 59.6 to 56.4, a decrease which suggests a heat effect but does not demonstrate it. The over-all heat damage, averaging 20.1%, is highly significant statistically (P = 0.00044).

The effect of the subsequent toasting of the corn flakes was measured in another nitrogen balance study not reported in table 1. During toasting the protein digestibility increased on an average from 73.8 to 79.8%, the reality of which is attested by the low probability of a chance outcome, 0.00083. The biological value did not change significantly, averaging 55.1 for the untoasted product and 54.3 for the toasted, P

being equal to 0.24. The change in net utilization, + 6.1%, is of doubtful significance (P = 0.069), though rather strongly suggestive of a favorable effect of the toasting process.

The effect of roasting on the protein value of beef

Two cuts of beef round, 3 in. thick, were taken from symmetrical parts of the same carcass. One was prepared directly for protein assay, while the other was roasted in the food laboratories of the Department of Home Economics by a standard method. The meat was roasted for 5 hours in an oven at 300°F. The medium internal temperature was 160°F. with the thermometer inserted 1.8 in. Both raw and roasted cuts were trimmed of visible fat, ground, dried at a temperature approximating 122°F., reground and then extracted with ether.

The average results of the nitrogen balance studies are given in the last line of table 1. The average digestibilities of protein for the raw and roasted beef and the average biological values are very nearly the same, and statistical analysis of paired differences revealed no grounds for presuming that the method of roasting employed modified appreciably the nutritive value of the protein (nitrogen) for growing albino rats.

Previous reports on the effect of various methods of cooking meat on the nutritive value of the contained nitrogen are cited by Poling, Schultz and Robinson ('44), who found some indications from their own work that roasting fresh pork shoulder may lower the protein efficiency ratio for growing rats when the meat is incorporated in experimental rations at a level of 9 to 10%. In the absence of a statistical analysis of the data it is difficult to judge the validity of this conclusion. As a supplement to a basal diet composed largely of yellow corn, fresh, cooked and cooked-cured meat (Wilder and Kraybill, '47) proved equally efficient in supplementing the lysine deficiency of the basal ration under the conditions of feeding employed. The cooking procedure consisted of heating the

meat in a retort for one hour at a temperature of 234 to 236°F.

The thiamine and pantothenic acid contents of some of the meals studied

In the hope of detecting a correlation between heat damage to the proteins of food products and the loss of thiamine and pantothenic acid brought about by heating, these vitamins were determined in many of the food products studied with

TABLE 2

The thiamine and pantothenic acid contents of the raw and heated meals

SAMPLE	THIAMINE	PANTOTHENIC ACID
	μg/gm	μg/gm
Peanut meal, solvent-extracted	12.0	34.5
Peanut expeller meal 1	7.3	32.5
Peanut expeller meal 2	7.0	37.0
Peanut expeller meal 3	5.1	29.5
Peanut expeller meal 4	5.5	30.5
Peanut expeller meal 5	8.7	38.5
Peanut expeller meal 6	7.4	36.5
Flaxseed (linseed)	6.2	8.5
Linseed meal, solvent-extracted	7.6	11.5
Linseed meal, expeller	2.4	13.0
Cottonseed flour, solvent-extracted	39.1	15.5
Cottonseed flour, hydraulic	16.3	12.0
Sunflower seed meal, solvent-extracted	36.4	12.0

reference to protein utilization by growing rats. The results are summarized in table 2. The assays were carried out under the supervision of Dr. B. Connor Johnson and by methods that he recommends in his manual (Johnson, '48). The thiamine was determined by a thiochrome method and the pantothenic acid by a microbiological method using *L. arabinosus* as the test organism. It should be noted that the latter method does not determine quantitatively the pantothenic acid bound in coenzyme A.

The thiamine values, more than pantothenic acid values, showed a response to heat, though the correlation with pro-

tein changes was poor, particularly for the peanut "expeller" meals. The destruction of thiamine in cottonseed flour by oil extraction by the hydraulic process was far greater than the decrease in protein value.

SUMMARY

The effect of heat as applied to food products during commercial processing and home cooking on the nutritive value of the food proteins for the growing rat has been studied on a number of foods by the nitrogen balance method developed in this laboratory. The samples compared were unheated, or very mildly heated as in a solvent-extraction method, and heated to high temperatures by autoclaving in the laboratory (sunflower seed meal), subjection to oil extraction by the expeller process (peanut and linseed meals) or the hydraulic process (cottonseed flour), or subjection to a process of flaking and toasting (corn). As an example of home cooking, the effect of a standard method of roasting on the proteins of beef was ascertained. In all cases, the unheated or mildly heated sample and the highly heated sample were obtained from the same raw material.

During these heating processes the digestibility of the proteins of sunflower seed meal, cottonseed flour and corn was definitely decreased by amounts ranging from 2.5 to 14.2 percentage units. The biological value of the proteins of peanut meal, sunflower seed meal, and cottonseed flour was also definitely decreased in these processes.

The percentage of total heat damage was highest for corn (20) and peanut meal (18), intermediate for sunflower seed flour (10) and cottonseed flour (11); no heat damage was demonstrated in the roasting of beef. For flaxseed (linseed), heat exerted a favorable effect on protein utilization (7%), especially evident in improved protein digestibility.

The heat processes studied always decreased the thiamine content of those foods subjected to assay, but the pantothenic acid content was not impaired in the peanut meals judged by

an assay method that does not measure the amount of the vitamin contained in coenzyme A.

ACKNOWLEDGMENTS

The authors wish to acknowledge the invaluable assistance of Mr. Ezra Levin of the VioBin Corporation, Monticello, Ill., who, at considerable expense and trouble, secured for us the peanut meals and linseed meal used in these studies, and prepared for our use the solvent-extracted peanut flour, cottonseed flour, linseed meal, and sunflower seed meal. The raw and processed corn samples were supplied by the Lauhoff Grain Co., of Danville, Ill., through the courtesy of Mr. E. R. Darling.

The assistance of the following staff members in carrying out the nitrogen balance studies is gratefully acknowledged: Mrs. Barbara C. Hamilton, Mr. W. T. Haines, and Mr. George D. Wood.

LITERATURE CITED

Buss, L. W., and V. R. Goddard 1948 Effect of heat upon the nutritive values of peanuts. I. Protein quality. Food Res., *13:* 506–511.

Fisher, R. A. 1944 Statistical Methods for Research Workers. Edinburgh and London, 9th ed., p. 350.

Jacobs, M. B. 1944 The chemistry and technology of food and food products. New York City, Interscience Publishers, Inc., Vol. II, p. 503.

Johnson, B. C. 1948 Methods of Vitamin Determination. Burgess Publ. Co., Minneapolis, Minn., p. 109.

Mitchell, H. H. 1924 A method of determining the biological value of protein. J. Biol. Chem., *58:* 873–903.

———— 1944 Determination of the nutritive value of the proteins of food products. Ind. Eng. Chem., Anal. Ed., *16:* 696–700.

Mitchell, H. H., E. W. Burroughs and J. R. Beadles 1936 The significance and accuracy of biological values of proteins computed from nitrogen metabolism data. J. Nutrition, *11:* 257–274.

Olcott, H. S., and T. D. Fontaine 1941 The effect of autoclaving on the nutritive value of the proteins in cottonseed meal. Ibid., *22:* 431–437.

Osborne, T. B., L. B. Mendel and E. L. Ferry 1919 A method of expressing numerically the growth-promoting value of proteins. J. Biol. Chem., *37:* 223–229.

Poling, C. E., H. W. Schultz and H. E. Robinson 1944 The retention of the nutritive quaility of beef and pork muscle proteins during dehydration, canning, roasting and frying. J. Nutrition, *27:* 23–34.

Student 1908 The probable error of a mean. Biometrika, *6:* 1–25.

Wilder, O. H. M., and H. R. Kraybill 1947 Effect of cooking and curing on lysine content of pork luncheon meat. J. Nutrition, *33:* 235–241.

ASSAY OF DRY PROTEINS BY RAT REPLETION METHOD. NUTRITIVE VALUE AND AMINO ACID COMPOSITION OF SIX REFERENCE PROTEINS

DOUGLAS V. FROST AND HARRY R. SANDY
Abbott Laboratories, North Chicago, Illinois

ONE FIGURE

(Received for publication July 28, 1949)

The rat repletion method of assaying protein value has been well established by Cannon and his co-workers at the University of Chicago (Wissler, Steffee, Woolridge, Benditt and Cannon, '47). The qualitative and quantitative requirements of amino acids for tissue repletion (Frazier et al., '47; Cannon, '48) and for maintenance (Wissler et al., '48; Frazier et al., '49) have been reported. The gain in weight of protein-depleted rats fed amino acid mixtures was reported to be directly parallel to changes in carcass protein (Benditt et al., '48; Steffee et al., '49).

The rat repletion method was studied in this laboratory as a means of assaying the essential amino acid value of liquid protein hydrolysates (Frost and Sandy, '48a, '48b). It was subsequently found to be convenient for determining the nutritive value of amino acid mixtures in solution (Frost and Sandy, '49).

The present study deals particularly with the assay of whole powdered protein preparations. As in the assay of protein hydrolysate solutions, the nitrogenous supplement was fed at controlled critical levels of nitrogen apart from

the remainder of the diet. Separate feeding of the supplement is believed to offer certain advantages over incorporation in the diet, as will be brought out in the discussion section of the present paper. Six reference protein materials distributed to various laboratories by the Bureau of Biological Research of Rutgers University provided substances of especial interest for the study. Hawley, Murlin, Nasset and Szymanski ('48) described these 6 proteins in connection with studies on their biological value in humans.

TABLE 1

Non-protein diet (NP no. 10) for rat-repletion studies

COMPONENT	AMOUNT	COMPONENT	AMOUNT
	gm/100 gm		*mg/100 gm*
Sucrose	83	Thiamine HCl	0.6
Salts—U.S.P. (No. 1)	4	Riboflavin	1.2
$CaHPO_4 \cdot 2H_2O$	1	Nicotinamide	3.7
Agar	1.4	Pyridoxine HCl	0.6
Primex	4.2	d (+) Ca pantothenate	5.0
Corn oil	4.6	*p*-aminobenzoic acid	0.6
Cod liver oil	1.4	Folic acid	0.6
Choline chloride	0.15	Biotin	0.04
Inositol	0.1	Menadione	0.4
Liver fraction "O" (Wilson)	0.1	$CuSO_4 \cdot 5H_2O$	4.0
Ascorbic acid	0.01	$ZnSO_4 \cdot 7H_2O$	4.0
$MnSO_4 \cdot 2H_2O$	0.01	$CoCl_2 \cdot 6H_2O$	0.2

EXPERIMENTAL

Weanling male rats were raised to a weight of 180 to 220 gm on a stock colony diet preparatory to depletion on a non-protein diet. Diets and water were fed ad libitum throughout the assays. The composition of the non-protein diet (diet NP 10) is shown in table 1. This diet differs from that previously used (Frost and Sandy, '48a, '48b) because of an increased amount of phosphorus and calcium and through the addition of copper, cobalt, manganese, folic acid, biotin, *p*-aminobenzoic acid, inositol, menadione, and a low nitrogen liver fraction as a source of vitamin B_{12}. The diet supplies about 4.2 cal. per gram and contains 0.03% N. Rats have been carried

through 5 successive periods of depletion and repletion on this diet without apparent injury. It is our practice, however, in using rats for repeated assays, to return them to a stock diet at intervals for equilibration.

Near-adult male rats lose an average of 20 to 25% of body weight during the initial 12-day depletion period on a non-protein diet. The rats are ordinarily prepared for assay of either liquid amino acid solutions or dry proteins by a trial period wherein a standard 5% fibrin hydrolysate [1] is fed for three days. Animals which do not learn to drink the full allotment of about 40 ml during this period may be eliminated. Skim milk has been successfully tried in place of the fibrin hydrolysate in the drinking trial period; no water is offered during this time. Animals prepared in this way show great avidity for either dry protein or liquid hydrolysates and seldom fail to consume the allotted supplements. In the assays herein reported, all supplements were consumed completely.

The Rutgers reference proteins were assayed at two levels of nitrogen, i.e., 0.12 and 0.24 gm N per rat per day. These levels of nitrogen, using the protein conversion factor 6.25, correspond to levels of protein in the diet of about 7 and 14%. The upper level is approximately the minimum level of feeding at which proteins of the best biological value elicit a maximum response. The lower level is a highly critical level of nitrogen intake, though somewhat above the absolute amino acid minima required for net repletion of tissue proteins to proceed. Nitrogen balance studies in selected cases served to demonstrate the sharp change from nitrogen loss to strong positive N balance during the shift from depletion to repletion. Benditt et al. ('48) have clearly demonstrated the relation of tissue protein synthesis to body weight gain at various levels of N intake in adult protein-depleted rats, so that further elaboration of our data along this line was not undertaken.

[1] Aminosol, 5%, in dextrose, 5%, was used on the basis of convenience. Other commercial hydrolysates may be suitable.

Groups of 5 rats were used for assay. In a few instances the same groups of animals were used in repeat assays of the same protein in order to establish the duplicability of results. Purified beef blood fibrin and lactalbumin were used as comparison standards. Results are shown in table 2.

TABLE 2

Rat repletion responses to selected protein materials at two controlled levels of feeding

PROTEIN SUPPLEMENT[1]	LEVEL OF FEEDING SUPPLEMENT			
	0.12 gm N/day		0.24 gm N/day	
	Ave. 12-day gain and std. error	Range	Ave. 12-day gain and std. error	Range
	gm	*gm*	*gm*	*gm*
Egg albumin	38 ± 3.5	30–46	80 ± 4.9	72–98
Whole egg (defatted)	33 ± 1.4	30–36	66 ± 3.9	53–81
Beef muscle (defatted)	31 ± 1.4	26–33	46 ± 3.5	39–53
Casein	31 ± 2.9	23–37	45 ± 3.7	35–52
Peanut flour	8 ± 6.3	1–13	32 ± 2.7	25–41
Wheat gluten	9 ± 2.3	5–14	19 ± 2.4	11–26
Fibrin, beef blood	35 ± 2.3	34–45	59 ± 0.7	58–60
Lactalbumin	44 ± 2.1	36–52	60 ± 4.5	45–70

[1] The first 6 protein samples are the reference samples distributed for collaborative trial by the Bureau of Biological Research of Rutgers University and described by Hawley et al. ('48). The fibrin is washed and dried beef blood fibrin, which contains 15% N. The lactalbumin is Borden lactalbumin 1542, which contains 12.5% N.

Amino acid analyses

The Rutgers samples were subjected to microbiological and chemical analysis for essential amino acids so that a direct correlation could be made with the rat repletion response. One-gram samples of the proteins were refluxed with 8N HCl for 8 hours to provide complete hydrolysates for microbiological analysis by the methods of Stokes et al. ('45) and Gunness and associates ('46) for leucine, isoleucine, phenylalanine, threonine, histidine, lysine, arginine, valine, methionine and tyrosine. Tryptophan was determined chemically by the method of Graham et al. ('47). The intact proteins were analyzed for nitrogen by the macro-Kjeldahl procedure

with a mercury sulfate catalyst, for ash, and for moisture. Results of all analyses are shown in table 3. For convenience in comparison, the amino acid values were calculated on the basis of 16% N for all proteins.

As will be seen in the table, the total essential amino acid content of peanut flour and gluten is much lower than for other proteins. There are clear-cut limiting deficiencies of methionine and lysine, respectively, in these two proteins. Limiting amino acids in the other proteins are less apparent.

TABLE 3

Amino acid analyses of Rutgers reference proteins
(Calculated to basis of 16% N)

	EGG ALBUMIN	CASEIN	WHOLE EGG	PEANUT FLOUR	WHEAT GLUTEN	BEEF MUSCLE
	%	%	%	%	%	%
Arginine	5.1	3.9	6.3	8.6	3.1	7.5
Histidine	2.7	3.4	2.2	2.1	2.0	4.4
Isoleucine	5.8	5.8	5.9	3.4	4.2	4.8
Leucine	9.5	9.8	9.1	5.7	6.5	9.2
Lysine	4.5	7.0	5.7	2.5	1.6	10.0
Methionine	4.1	3.4	3.5	0.6	1.3	3.0
Cystine	3.3	0.4	2.2	1.7	2.7	1.2
Phenylalanine	6.8	5.8	6.0	4.3	4.9	4.9
Tyrosine	4.0	5.5	3.4	2.8	2.8	2.5
Threonine	4.9	4.6	5.2	2.3	2.6	5.6
Tryptophan	2.0	1.4	1.9	1.5	1.3	1.9
Valine	7.9	7.4	7.9	3.7	3.7	6.0
Total	60.6	58.4	59.3	39.2	36.4	61.0
Total Nitrogen	11.8	13.22	12.16	10.16	12.65	15.65
Ash	6.2	0.9	4.4	3.9	0.6	4.2
Moisture	8.9	8.1	5.1	3.9	7.0	3.2

Technique for 5-day routine assay

A convenient assay schedule which places all weighings and supplements in the 5-day work week, and which provides for repeated use of rats, is now in use in these laboratories for routine control of an intravenous fibrin hydrolysate.[2] Ani-

[2] See footnote 1, page 429.

mals prepared as described above are fed test materials for 5 days at a level of 0.2 gm N per day. The rats are fed a stock diet for two days over the week-end for maximum repletion. They are then returned to the non-protein diet for 7-day depletion, after which the assay cycle is repeated. Groups of 5 male rats are used and the assays are controlled by a reference protein, lactalbumin. Water is fed ad libitum. The procedure is applicable to routine control of dry protein materials or of liquid protein hydrolysates.

A curve representing the average weight changes of 5 rats from a control group used through 8 successive assays is shown in figure 1. The average response of the 5 rats, together with the range, is shown. A step-wise regular increase in weight occurs under these conditions despite the periodic depletions. As will be observed, the magnitude of the response gradually becomes somewhat less. The assays are judged in reference to the parallel response of the positive control group, so that this variable is controlled. The assay groups which ran parallel to the group shown in figure 1 occasionally gained an average of only about 12 gm during the 5-day assay period. Lesser gains such as this did not appear to affect subsequent responses. The two-day period on a stock diet is particularly useful in permitting repeated use of the animals because it provides a strong equalizing influence.

DISCUSSION

The relative nutritive value of individual proteins for growth, repletion or maintenance is primarily related to their essential amino acid composition. There is also a question of the availability of certain amino acids in raw and processed proteins. None of the proteins presently studied was heated above about 80°C. (Hawley et al., '48). The wheat gluten was made from agenized flour, which has no apparent toxic effect on rats. In general, the proteins studied are considered unchanged from their native state as regards nutritive value.

Fair agreement in the relative rating of the proteins obtains between our results and those of Hawley et al. ('48) and Murlin et al. ('48). It may be pointed out that the studies of the Rochester group were involved with maintenance, requirements for which are quantitatively different than those for repletion. The relatively high requirements of the rat for the sulfur amino acids may account in part for the standout

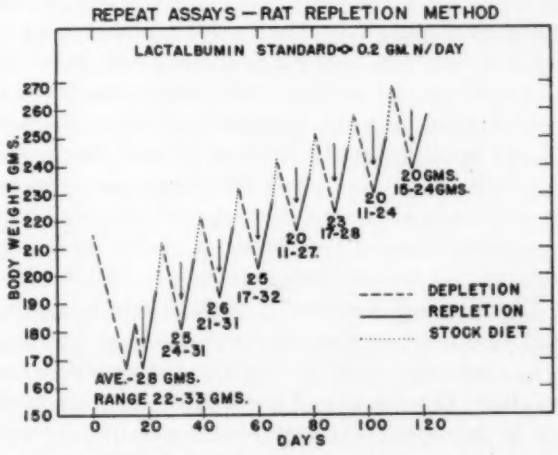


Fig. 1 Average weight changes of 5 rats on lactalbumin as a standard in manufacturing control assay. First part of curve shows 12-day depletion on non-protein diet followed by three-day "drink trial" and three-day redepletion. Subsequent assay cycles consist of lactalbumin (5 days' repletion), stock diet (2 days) and redepletion (7 days). Averages and ranges of weight gain for each of the 8 assay periods are shown.

performance of egg albumin and lactalbumin in the present study over the other proteins high in biological value, particularly fibrin, beef muscle and casein.

Minimum daily requirement values for a maximum rate of repletion in the adult protein-depleted rat have been advanced on a tentative basis by Cannon ('48), as follows: tryptophan 13, phenylalanine 37, leucine 77, isoleucine 66, methionine 35, threonine 45, lysine 51, histidine 22 and valine 53 mg per rat per day. These values were established with a 16-amino acid

mixture by individually reducing the level of each of the essential amino acids, step-wise, to a point where a falling off in the magnitude of repletion response began to take place. According to Cannon, the values are tentative and are, therefore, subject to revision.[3] Also, the values apply precisely only to the 16-amino acid mixture patterned after casein which was used by the Chicago group.

One can calculate the adequacy of each of the essential amino acids as supplied by the various proteins at the 0.12 and 0.24 gm N levels of feeding in comparison with the above minima. In spite of the obvious quantitative limitations, there is good correlation between the nutritive value as herein determined and the calculated adequacy of essential amino acids supplied by the respective proteins. When the necessary calculations are made, it is seen that egg albumin and whole egg contain good patterns of the essential amino acids which approach minimum requirements even at the 0.12 gm N level of feeding. The limiting deficiency of both whole egg and egg albumin at this level appears in the isoleucine content, which supplies in each case only 70% of the requirement for maximum repletion. Obviously, at the higher level of feeding no deficiency is presented by these proteins and maximum rates of repletion are possible. Again, for defatted beef muscle at the lower feeding level 44% of the methionine need and 47% of the isoleucine need are supplied, which would appear to account for the lesser response to this protein. At the lower feeding level the limiting deficiencies for the remaining proteins appear as follows: for casein, methionine 64% and tryptophan 69%; for peanut flour, methionine 13% and lysine 32%; and for wheat gluten, lysine 20% and methionine 26%.

The primary deficits of methionine in peanut flour and of lysine in gluten are well-known. It is of interest to note that the second limiting deficiency for peanut flour appears to be lysine and for wheat gluten, methionine. Little or no supple-

[3] It was learned by personal communication with Dr. Paul R. Cannon that repletion minima revised slightly from those shown above are now in press, together with minimum requirements for maintenance of nitrogen balance.

mentary effect would be predicted, therefore, for a mixture of these proteins. In assessing methionine as a limiting amino acid, one must consider the attendant cystine content, and also the factor of utilization of methionine. The low cystine content of casein is well known and there is evidence in this laboratory (Frost and Sandy, '48b) that the methionine of casein is not completely utilized.

Final assessment of the over-all nutritive value of proteins must also be concerned with metabolic interrelationships having to do with the balance among amino acids themselves and with other nutrients. Such well recognized interrelationships as that between tryptophan and niacin may require increasing attention, with regard to the adequacy of the diet in factors which outwardly appear to be quite unrelated.

Mitchell and Block ('46) and Block and Mitchell ('46) have compared the amino acid composition of a large number of food proteins with that of whole egg as a standard of nutritive excellence. The chemical scores thus obtained are in fair general agreement with the results of growth and nitrogen balance assays in rats. Such methods of chemical scoring are of considerable value in revealing the nature of amino acid deficiencies in proteins. The meaningfulness of the particular reference standard chosen, the correctness of the amino acid analyses, lack of knowledge of species requirements, and the effect of processing on protein value are, however, all questions with which the analyst and nutritionist must cope in any such appraisal of relative protein values. Because these questions involve many variables which are not amenable to control, it is expected that animal assay methods will continue in vogue for some time and that methods of chemical scoring will provide valuable supplementary information.

The repletion method may be considered of particular value in measuring the adequacy of proteins to correct conditions of protein deficit such as occur during growth, pregnancy and lactation, and convalescence from illness or injury. The demands for essential amino acid adequacy and balance are

greatest during these periods, and are fairly well simulated by the conditions of the repletion method. The rat growth method is probably equally valuable, but requires longer time and does not allow repeated use of animals. A criticism of both methods as applied to protein value for human nutrition is pointed up by the difference in species requirements for methionine (Cox et al., '47). Where a limitation in total sulfur amino acids for the rat is known to exist, as in casein, correction can be made by incorporating an appropriate amount of methionine in the basal diet.

Although it is common practice in assaying food substances such as vitamins to feed them separately, proteins have generally been incorporated directly in the diet. Separate feeding of the supplement appears to us to offer advantages in respect to measurement of intake, and also to offer certain theoretical advantages pertaining to the experimental results and the interpretation thereof. In the present method the supplement is fed separately from the remainder of the diet, which is supplied ad libitum. Under these conditions, the amount of non-protein diet consumed is thought to be a reflection of appetite, which in turn reflects the well-being of the animal. The results of assays on the same proteins and different proteins at various critical levels of nitrogen are in support of this hypothesis. The quality of a protein may be excellent, but if it is not fed at an optimum level the supply will itself automatically limit the appetite and hence the food consumption. Proteins of poor quality, even when fed at relatively high levels, may still not meet the full quantitative requirements for a maximum rate of repletion, which results in a limited intake of total food. Hegsted and Haffenreffer ('49) have recently presented evidence and a rationale in support of ad libitum feeding, particularly in regard to calorie intake. Feeding methods which limit the amino acid nitrogen intake without limiting the intake of other food substances are based on this line of reasoning.

The ranges as well as the standard errors of the repletion responses to the reference proteins are shown in table 2 as

an aid in considering the mathematical significance of the data. In general the standard errors are small considering that only 5 animals were used in each group. The variation in response was greatest for peanut flour and wheat gluten fed at the lower nitrogen level. The latter conditions are most rigorous and it would be expected that even small differences in protein reserves might affect the results between animals. Although such information is of interest, the object of the investigation was not to develop the most rigorous conditions to reveal protein deficits, but rather to develop a simplified method which would reliably classify a wide range of proteins as to essential amino acid value, with a minimum of variance within assay groups.

The "t" test of significant difference was applied to the data of table 2 and a P value of 0.05 or less was considered statistically significant. By this criterion it was found that the difference in response between egg albumin and whole egg is significant at the 0.24 gm N level but not at the 0.12 gm N level. The differences between whole egg and beef muscle (or casein) and between peanut flour and gluten were highly significant at the 0.24 gm N level but not at all significant at the 0.12 gm N level. It would appear from this that the 0.24 gm N level allows better resolution of differences in nutritive value than does the 0.12 gm N level. The level of 0.2 gm N per day was chosen as advantageous for routine control purposes since it provides a good determination of nutritive value at a level of feeding where the ration is readily consumed.

SUMMARY

Six reference proteins obtained from Rutgers University as part of a collaborative test program, plus fibrin and lactalbumin, were assayed by the rat repletion method as supplements separate from a non-protein diet. Feeding levels of 0.12 and 0.24 gm N per rat per day were studied. The repletion response to the reference proteins was roughly in the

following order: egg albumin, defatted whole egg, defatted beef muscle and casein about on a par, peanut flour, and wheat gluten. Fibrin and lactalbumin compared favorably with the egg proteins. The weight responses were related to the essential amino acid composition of the reference proteins. The advantages and limitations of the method are discussed, together with the significance of the data obtained.

A convenient method for routine control of the protein value, wherein rats are reused many times, is described.

ACKNOWLEDGMENTS

We wish to thank H. C. Spruth and Albin Junnila for data relating to the 5-day routine assay; Eleanor Willerton and Shirley Hansen for microbiological amino acid assays; and E. O. Krueger for chemical amino acid determinations. We are grateful to Paul R. Cannon for his interest and helpful counsel at several stages of this study.

LITERATURE CITED

BENDITT, E. P., E. M. HUMPHREYS, R. W. WISSLER, C. H. STEFFEE, L. E. FRAZIER AND P. R. CANNON 1948 The dynamics of protein metabolism. I. The interrelationship between protein and calorie intakes and their influence upon the utilization of ingested protein for tissue synthesis by the adult protein-depleted rat. J. Lab. Clin. Med., *33:* 1.

BLOCK, R. J., AND H. H. MITCHELL 1946–47 The correlation of the amino acid composition of proteins with their nutritive value. Nutrition Abstr. and Rev., *16:* 249.

CANNON, P. R. 1948 Some pathologic consequences of protein and amino acid deficiencies. Charles C. Thomas, Publisher, Springfield, Ill.

COX, W. M., A. J. MUELLER, R. ELMAN, A. A. ALBANESE, K. S. KEMMERER, R. W. BARTON AND L. E. HOLT 1947 Nitrogen retention studies on rats, dogs and man; the effect of adding methionine to an enzymic casein hydrolysate. J. Nutrition, *33:* 437.

FRAZIER, L. E., R. W. WISSLER, C. H. STEFFEE, R. L. WOOLRIDGE AND P. R. CANNON 1947 Studies in amino acid utilization. I. The dietary utilization of mixtures of purified amino acids in protein-depleted adult albino rats. Ibid., *33:* 65.

FRAZIER, L. E., R. L. WOOLRIDGE, C. H. STEFFEE AND E. P. BENDITT 1949 Minimum daily requirements of indispensable amino acids for maintenance of adult rats. Fed. Proc., *8:* 355.

FROST, D. V., AND H. R. SANDY 1948a Method for determining amino acid adequacy of liquid protein hydrolysates in rats. Proc. Soc. Exp. Biol. Med., *68:* 51.

———— 1948b Partial acid hydrolysates of proteins. VI. Assay of liquid protein hydrolysates in protein-depleted rats. J. Biol. Chem., *175:* 635.

———— 1949 Rat-repletion studies with amino acid solutions. Role of non-essential amino acids. Fed. Proc., *8:* 383.

GRAHAM, C. E., E. P. SMITH, S. W. HIER AND D. KLEIN 1947 An improved method for determination of tryptophan with *p*-dimethyl amino benzaldehyde. J. Biol. Chem., *168:* 711.

GUNNESS, M., I. M. DWYER AND J. L. STOKES 1946 Extension of the uniform assay method for the 10 essential amino acids to include tyrosine. Ibid., *163:* 159.

HAWLEY, E. E., J. R. MURLIN, E. S. NASSET AND T. A. SZYMANSKI 1948 Biological value of 6 partially-purified proteins. J. Nutrition, *36:* 153.

HEGSTED, D. M., AND V. K. HAFFENREFFER 1949 Calorie intakes in relation to the quantity and quality of protein in the diet. Am. J. Physiol., *157:* 141.

MITCHELL, H. H., AND R. J. BLOCK 1946 Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem., *163:* 599.

MURLIN, J. R., T. A. SZYMANSKI AND E. C. NASSET 1948 Creatinine nitrogen percentage as a check on the biological values of proteins. J. Nutrition, *36:* 171.

STEFFEE, C. H., R. W. WISSLER, E. M. HUMPHREYS, E. P. BENDITT, R. L. WOOLRIDGE AND P. R. CANNON 1949 Determination of minimal daily essential amino acid requirements in protein-depleted male albino rats. Fed. Proc., *8:* 369.

STOKES, J. L., M. GUNNESS, J. M. DWYER AND M. C. CASWELL 1945 Microbiological methods for the determination of amino acids. II. A uniform assay for the 10 essential amino acids. J. Biol. Chem., *160:* 35.

WISSLER, R. W., C. H. STEFFEE, L. E. FRAZIER, R. L. WOOLRIDGE AND E. P. BENDITT 1948 Studies in amino acid utilization. The role of the indispensable amino acids in maintenance of the adult albino rat. J. Nutrition, *36:* 245.

WISSLER, R. W., C. H. STEFFEE, R. L. WOOLRIDGE, E. P. BENDITT AND P. R. CANNON 1947 The evaluation of protein hydrolysates. J. Am. Diet. Assn., *23:* 841.

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc. for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojurning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1950 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 15, 1950.

H. E. Carter
University of Illinois
Urbana, Illinois

Chairman, Nominating Committee

MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1950 Award of $1000.00 established by Mead Johnson and Company to promote researches dealing with the B-complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1950.

The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1949 must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

W. H. Sebrell, Jr.
Experimental Biology and Medicine Institute
National Institutes of Health
Bethesda, Maryland

Chairman, Nominating Committee

BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1950 Award of $1000.00 and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1950. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompnaied by such data relative to the nominee and his research as will facilitate consideration for the award.

L. A. MAYNARD
Cornell University, Ithaca, New York

CHAIRMAN, NOMINATING COMMITTEE